"Extended Spectrum Beta lactamases screening in Escherichia coli and Klebsiella isolates and confirmation by molecular method"

Dissertation submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

In partial fulfilment of the regulations for the award of the degree of

M.D BRANCH – IV

MOCROBIOLOGY

SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES
KULASHEKARAM

THE TAMILNADU DR. M.G.R MEDICAL UNIVERSITY CHENNAI, INDIA

APRIL 2017

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This to certify that the dissertation entitled **"EXTENDED SPECTRUM BETA LACTAMASES SCREENING IN ESCHERICHIA COLI AND KLEBSIELLA ISOLATES AND CONFIRMATION BY MOLECULAR METHOD"** is a bonafide work done by **DR. J. JASMINE GNANA SUTHA** from **SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES, KULASEKHARAM** in partial fulfillment of the University rules and regulations for award of degree of **Doctor of Medicine in Microbiology (Branch-IV)** of Tamil Nadu Dr. M.G.R. Medical University.

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I **DR. J. JASMINE GNANA SUTHA**, solemnly declare that dissertation titled, **"EXTENDED SPECTRUM BETA LACTAMASES SCREENING IN ESCHERICHIA COLI AND KLEBSIELLA ISOLATES AND CONFIRMATION BY MOLECULAR METHOD"** is a confide and genuine research work carried out by me in the department of Microbiology, Sree Mookambika Institute of Medical Sciences Kulashekaram, during 2013-2016 under the guidance and supervision of Professor **DR. N. PALANIAPPAN**, Department of Microbiology. Sree Mookambika Institute of Medical Sciences, Kulashekaram, and Professor **Dr. P. INDU M.D,** Head of the Department, Deportment of Microbiology, Sree Mookambika Institute of Medical Sciences, Kulashekaram.

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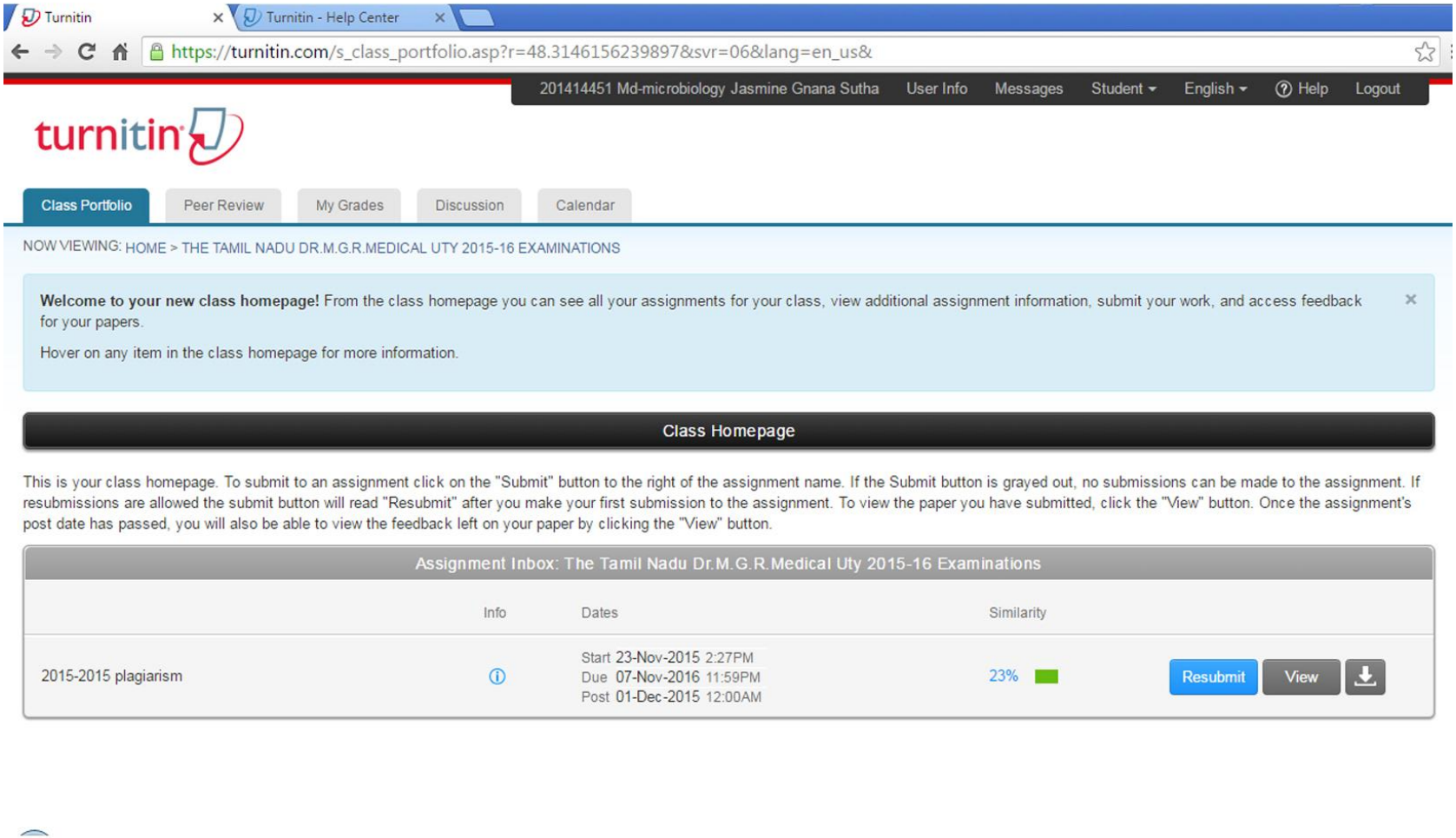
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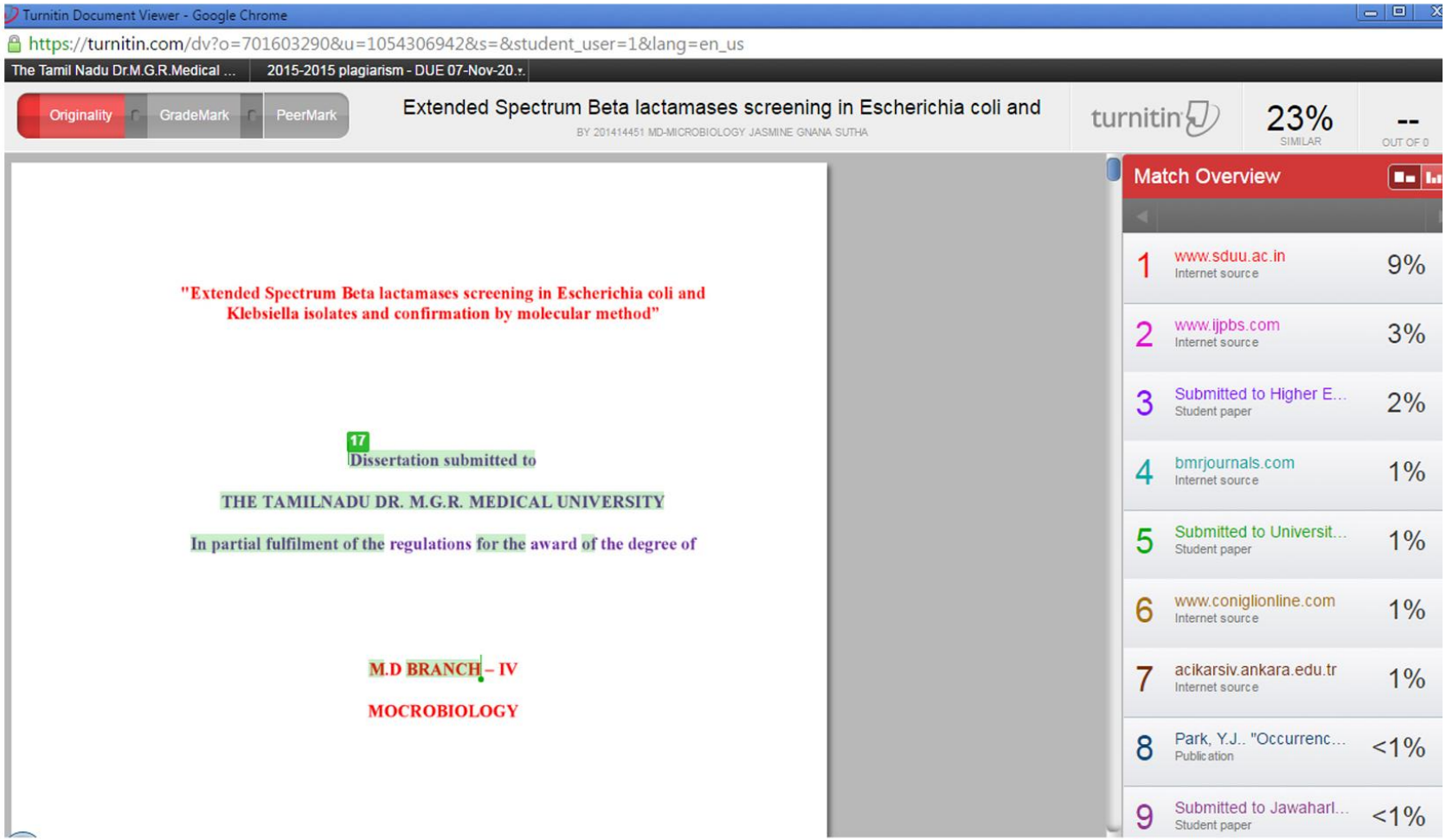
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Dr. J. Jasmine Gnana Sutha

CONTENTS

Introduction

INTRODUCTION

Problem discovery

Antimicrobial-resistance is emerging worldwide at an alarming rate among Gram-negative bacteria causing both community-acquired and hospital-acquired infections.[1,2] One of the most important emerging resistance profiles in bacteria belonging to the Enterobacteriaceae family corresponds to resistance to extended-spectrum β-lactams (ESBLs).[2,3,4] Extended spectrum β-lactamases are β-lactamase enzymes that have the ability to hydrolyze penicillins, extended-spectrum cephalosporins (oxyimino-β-lactams), and aztreonam, but not carbapenem or cephamycin antibiotics. ESBLs are reported worldwide among different bacterial species, including Enterobacteriaceae and non-fermentative Gram-negative bacteria such as *Pseudomonas* and *Acinetobacter* species.[72]

The major reservoir for such bacteria is the gastrointestinal tract.[5] Preceding gastrointestinal colonization by antimicrobial resistant bacteria has been associated with subsequent infection.[6]A study in America (USA) has emphasized the importance of identifying individuals carrying antimicrobial-resistant bacteria in both patient and healthy populations.[7] Another study reported that an increase in the proportion of carriage of antimicrobial-resistant bacteria in the community increases the risk that other individuals will also become carriers via human-to-human transmission.[8,9,10] In addition, the admission into hospital of patients harboring resistant bacteria increases the risk of other hospitalized patients

contracting an infection.[8,11,12] The actual prevalence of extended-spectrum β-lactamase (ESBL) producing organisms in healthy humans in the community settings is largely unknown and possibly under estimated.[13,14] The acquisition of antibiotic resistance genes at birth has been recently reported, with the mode of delivery affecting rates of acquisition,[15,16] however most studies on the community carriage of antimicrobial-resistant bacteria have been cross-sectional, and targeted adult populations.[14,17,18,21] In addition, few longitudinal birth-cohort studies describing the acquisition of ESBL producing organisms have been conducted worldwide including India.[22,23]

Associated factors influencing the emerging of ESBL

Several risk factors have been found to be which include prolonged hospitalization, prolonged ICU stay, multiple hospitalization, invasive procedures, immunosuppression and recent antibiotic treatment.[35,36,37] Other risk factors for ESBL rectal carriage are old age (older than 65 years), female sex and MRSA carriage.[37]

In the community, risk factors are still not clear but factors that enhance the spread of these organisms include lack of hygiene, overuse and over-the counter use of antibacterial drugs, and increased worldwide travel.[13]

Many studies have reported isolation of ESBL producing organisms from raw vegetables, fruits as well as food of animal origins. It is most likely that these animals might have acquired the ESBL producers from human contacts, at the same time, origin among themselves cannot be entirely ruled out. Although there are not

many reports indicting human acquisition of ESBL producers following consumption of contaminated animal food, the risk is always there.[13]

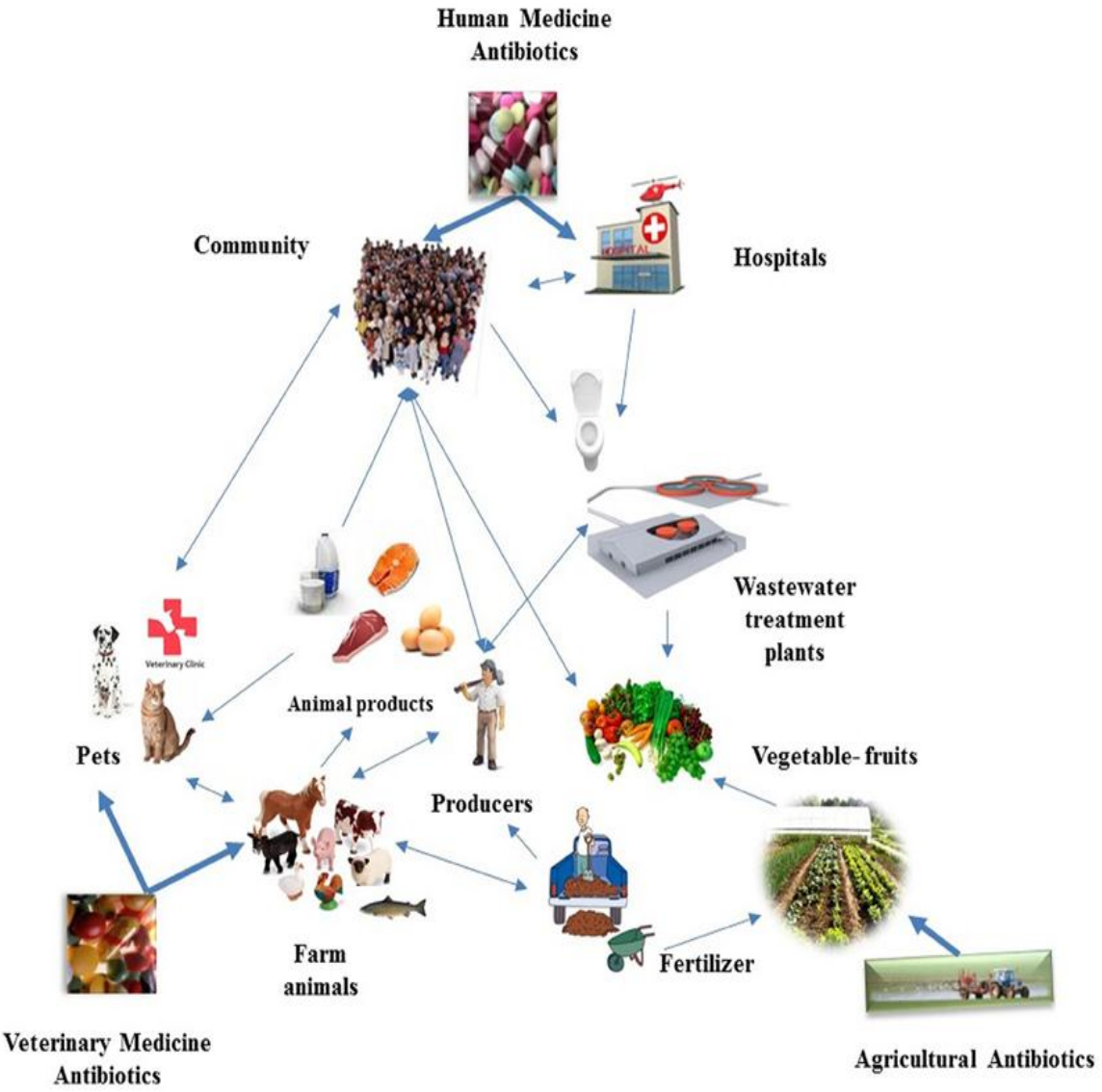


Figure 1. The environmental reservoirs of antimicrobial resistance.

Rationale and motivation

Antimicrobial-resistance in Gram-negative bacteria is a public health concern due to the limited availability of treatment options.[2,24,25] The prevalence of antimicrobial-resistant in Gram-negative bacteria that produce either extended-spectrum β-lactamases (ESBLs) has been increasingly reported over the past years, both in hospitals and communities worldwide.[3,4] The microorganisms producing ESBLs may be responsible for infections that are life-threatening, thus resulting in increased healthcare-associated costs, morbidity and mortality.[26] The first line drugs for the treatment of severe infections caused by ESBL-producing bacteria are the carbapenems,[27] however their efficacy is highly compromised by the emergence and spread of carbapenemase-producing bacteria worldwide.[28,29] The strains that produce ESBLs often carry resistance determinants for other classes of antimicrobial agents, and infections caused by these strains are associated with high mortality.[30,31,32] A major concern is the coexistence of multiple ESBL genes that has led to the emergence of organisms that are resistance to nearly all antibiotics.[33]

The prevalence of ESBL- producing organisms has been described in considerable detail in certain parts of the world.[30,32] The emergence of ESBLs has different epicenters, but they are now wide spread worldwide.[13] ESBLs have spread widely geographically, causing outbreaks in many parts of the world,[32] and this is likely to be the case with carbapenemases.[34]

In India also, their prevalence is increasingly reported in both hospital and community settings.[41, 42] Therefore, this review gives an overview of the prevalence of ESBL producers in India.

Therefore, this study aimed for the first time in our territory teaching hospital, to evaluate the prevalence of ESBL producing Esch.coli and Klebsiella in the clinical isolates, the present status of antibiotic resistance in Esch.coli and Klebsiella and to compare the results of phenotyping method with molecular typing for the successful treatment of the patient management and to apply proper control measures.

Aim and objectives

Aim and objectives

1. To evaluate the prevalence of ESBL producing Esch.coli and Klebsiella in the clinical isolates at Sree Mookambika Institute of Medical Sciences, Kulasekaram.
2. To study the present status of antibiotic resistance in Esch.coli and Klebsiella.
3. To compare the results of phenotyping method with molecular typing.

Review of literature

A review on the prevalence of extended-spectrum β-lactamase producing bacteria

The prevalence of ESBL producing organisms has been substantially described in Europe, North America, Asia and Africa.[13,34]In India also, their prevalence is increasingly reported in both hospital and community settings.[41,42] Therefore, this review gives an overview of the prevalence of ESBL producers in India.

An overview of antibiotic resistance

Discovery and occurrence of antibiotic resistance

Before discovery of penicillin by the British scientist Alexander Fleming in the late 1920s, infectious diseases were the leading cause of mortality worldwide.[43,44] The introduction of penicillin in the 1940s and together with vaccination resulted in improved life expectancy and reduced mortality rate due to infectious diseases during the 20th century.[43,45] However, soon after its introduction, penicillin-resistant *Staphylococcus aureus* was detected.[46] The resistance to penicillin was due to bacterial production of penicillinase, a β-lactamase enzyme capable of hydrolyzing penicillin.[46,47] Other antibiotics such as tetracycline, streptomycin and chloramphenicol were introduced in the late 1940s, likewise resistance to these antibiotics was also noted soon after their introduction.[48]Antibiotic resistance, defined as the ability of bacteria to resist the effects of antibacterial drugs, is one of the world most pressing public health threats.[48,49]Infections caused by multidrug-resistant (MDR) organisms are increasingly reported worldwide, with very limited treatment options.[1,2] Lack of

effective treatment of patients infected with MDR organisms has led to high mortality and morbidity rates.[50]The rate of novel antibiotic discovery is currently very low compared to the increasing resistance problem.[51] Among other factors, multiple mechanisms of resistance to antibiotics makes it difficult to develop or discover effective antibiotics.[50,51]

Mechanisms of antibiotic resistance

Resistance can be either an inherent trait of the bacterium or acquired.[52]A variety of bacterial species possess innate resistance through inherited mechanisms.[53]Some bacteria can mutate to highly resistant forms, however, this accounts for a small proportion of antimicrobial resistance.[48] Most bacteria become resistant to antibiotics through the getting hold of resistance-conferring deoxyribonucleic acid (DNA) from other sources.[53,54] Antimicrobial resistance genes can be carried on a plasmid, chromosome, or transposon; however, most of these genes are encoded on plasmids, which are self-replicating extra-chromosomal pieces of DNA in which may themselves carry transposons.[53,55]Transposons are non-self-replicating pieces of genetic material that can be easily exchanged between plasmid to plasmid, chromosome to chromosome, or between plasmid and chromosome.[55]Transposon also play a major role in disseminating resistance genes among bacterial species.[53]

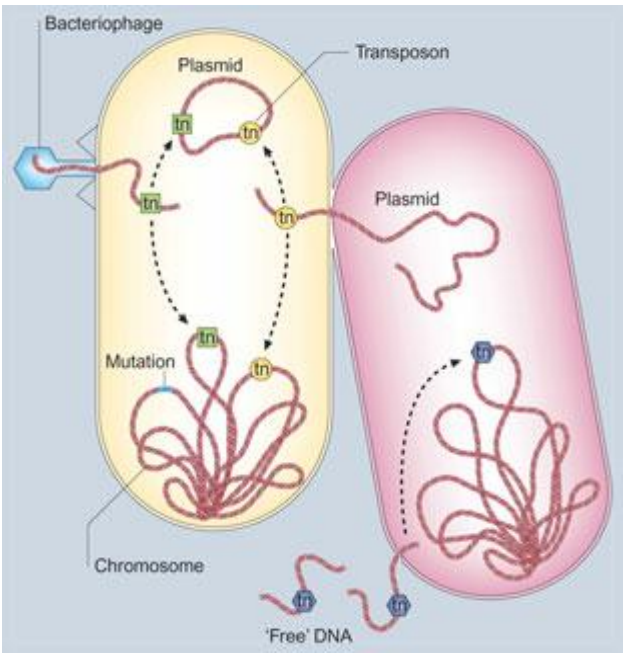


Figure 2. Transposon mechanism.

Resistance genes can be spread from one bacterial species to another by conjugation, transformation, or transduction. Plasmids can transfer genetic material through conjugation.[55] Transduction is a process in which a bacteriophage is needed for exchange of genetic material; whereas, transformation is the uptake of free DNA from the environment.[53]

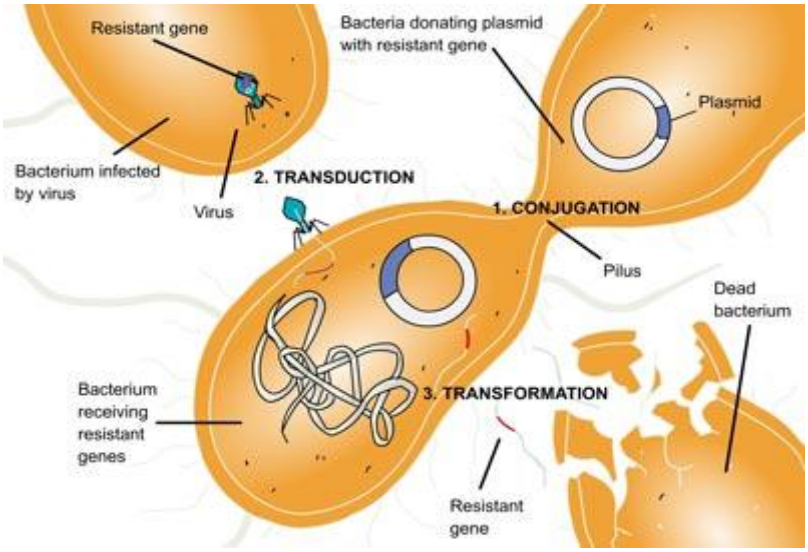


Figure 3. Mechanism of gene exchange.

Bacterial resistance to antibiotics can be caused by either the enzymatic modification of an antibiotic which renders it ineffective, the modification of its target site so that it is unable to exert its effect, active physical removal of the antibiotic from the cell, or by reduction of uptake into the bacterial cell due to changes in membrane permeability.[53] The main resistance mechanism is the enzymatic inactivation of an antibiotic and the most clinically important example of this is β-lactamase enzymes, the enzymes that hydrolyze β-lactam antibiotics.[56,57] This review will focus β-lactamase enzymes, which are the subject of this study.

β-lactams

Classification of β-lactams

β-lactams are a broad class of antibiotics that are widely used for treating bacterial infections.[58] All antibiotics in this class contain, in their molecular structure, a β-lactam ring which is a four membered lactam (figure 4)**.** β-lactam antibiotics include: the penicillins, cephamycins, cephalosporins, monobactams, carbapenems and β-lactamase inhibitors.[58]

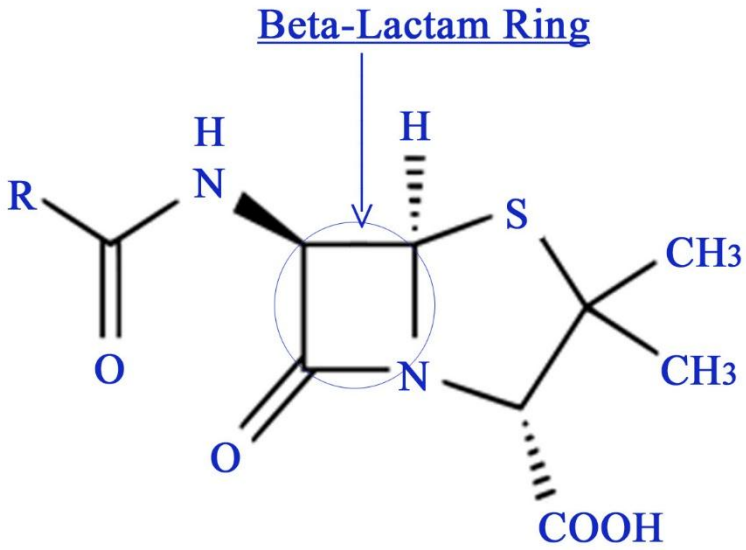


Figure 4. Core structure of penicillin and β-lactam ring.

The β-lactam ring is the active site of the β-lactam antibiotics that irreversibly bind to the penicillin binding proteins (PBPs) thus blocking their action of synthesizing a peptidoglycan layer. This leads to the weakened bacterial cell wall; and the bacterium eventually bursts.[59]

Penicillins

Penicillins are effective against most Gram-positive and some Gram-negative bacteria. Several types of penicillins exist; the natural penicillins (penicillin G), penicillinase resistant penicillins (e.g. methicillin and oxacillin) and amino-penicillins (e.g. ampicillin and amoxicillin). The first two types of penicillins have lethal effects on many Gram positive organisms, whereas amino-penicillins are effective against an extensive range of bacteria including some Gram-negative bacteria.[60,61]

Cephalosporins and cephamycins

Cephalosporins are subdivided into five generations including the closely related cephamycin compounds and together they establish a group of β-lactams called cephems. The 1st generation cephalosporins (e.g. cefazolin and cephalothin) were active pre-dominantly against Gram-positive cocci. The 2nd generation cephalosporins are more active against Gram-negative bacilli and are somewhat less effective against Gram-positive cocci. Unlike the 1st and 2nd generation cephalosporins, the 3rd generation cephalosporins have increased activity against both Gram-negative and Gram-positive organisms. Among the 4th generation cephalosporins, cefepime is the most commonly used antibiotic. The 4th generation cephalosporins have the similar activity against Gram-positive bacteria as the 1st generation cephalosporins; however, they have a greater activity against Enterobacteriaceae and *Pseudomonas aeruginosa* than the 3rd generation cephalosporins. The 5th generation cephalosporins (ceftaroline and ceftobiprole) notably improved activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-intermediate *Staphylococcus aureus* bacterias.[62,63]

Monobactams

A currently marketed monobactam is aztreonam, an antibiotic with a very good activity against aerobic and fastidious Gram-negative bacilli such as Enterobacteriaceae and *Pseudomonas aeruginosa* bacterias. Aztreonam has poor activity against anaerobes and Gram-positive organisms.[64]

Carbapenems

Carbapenems are normally mentioned to as the antibiotics of last alternative and are bactericidal for both Gram-negative and Gram-positive organisms. They are known to be the effective antibiotics against various multidrug-resistant Gram-negative bacilli. Carbapenems consist of imipenem, ertapenem, meropenem and doripenem.[65]

β-lactamase inhibitors

Clavulanic acid, tazobactam and sulbactam are β-lactamase inhibitors that inhibit a number of plasmid-mediated β-lactamases. They generally don't inhibit chromosomally-mediated β-lactamases. The combination of β-lactamase inhibitors

with β-lactams significantly increases spectrum of activity against many organisms containing plasmid-mediated β-lactamases.[66]

Mechanism of action and resistance

β- lactam antibiotics are bactericidal and inhibit the growth of sensitive bacteria by inhibiting the D-alanyl-D-alanine-transpetidases, the penicillin-binding proteins (PBPs) that facilitate the transpeptidation of peptidoglycan synthesis, an important component of the bacterial cell wall. Peptidoglycan, a murein, contains of amino acids and sugars that form an important part of the bacterial cell wall. β-lactam antibiotics are analogues of D-alanyl-D-alanine, the terminal amino acid residues on the precursor N-acetyl muramic acid/N-acetyl glucosamine (NAM/NAG) peptide subunits of the peptidoglycan layer. This structural resemblance allows the binding of β-lactams to the active site of PBPs. The β-lactam ring permanently binds to the Ser 403 residue of the PBP active site by acylation there by preventing the final transpeptidation or cross linking of budding peptidoglycan layer by the PBPs, disrupting synthesis of the bacterial cell wall followed by cell death. The main mechanisms of resistance to β-lactam antibiotics are decreased penetration of β-lactams due to the production of modified porins, loss of porins or a shift in the types of porins found in the outer membrane of Gram-negative bacteria, increased efflux from the cell through production of efflux pumps, or inactivation of β-lactams by chromosome- and /or plasmid-encoded β-lactamase

enzymes (Figure 5). Among the above-mentioned, the most common mechanism of resistance is the production of β-lactamases.[67,68]

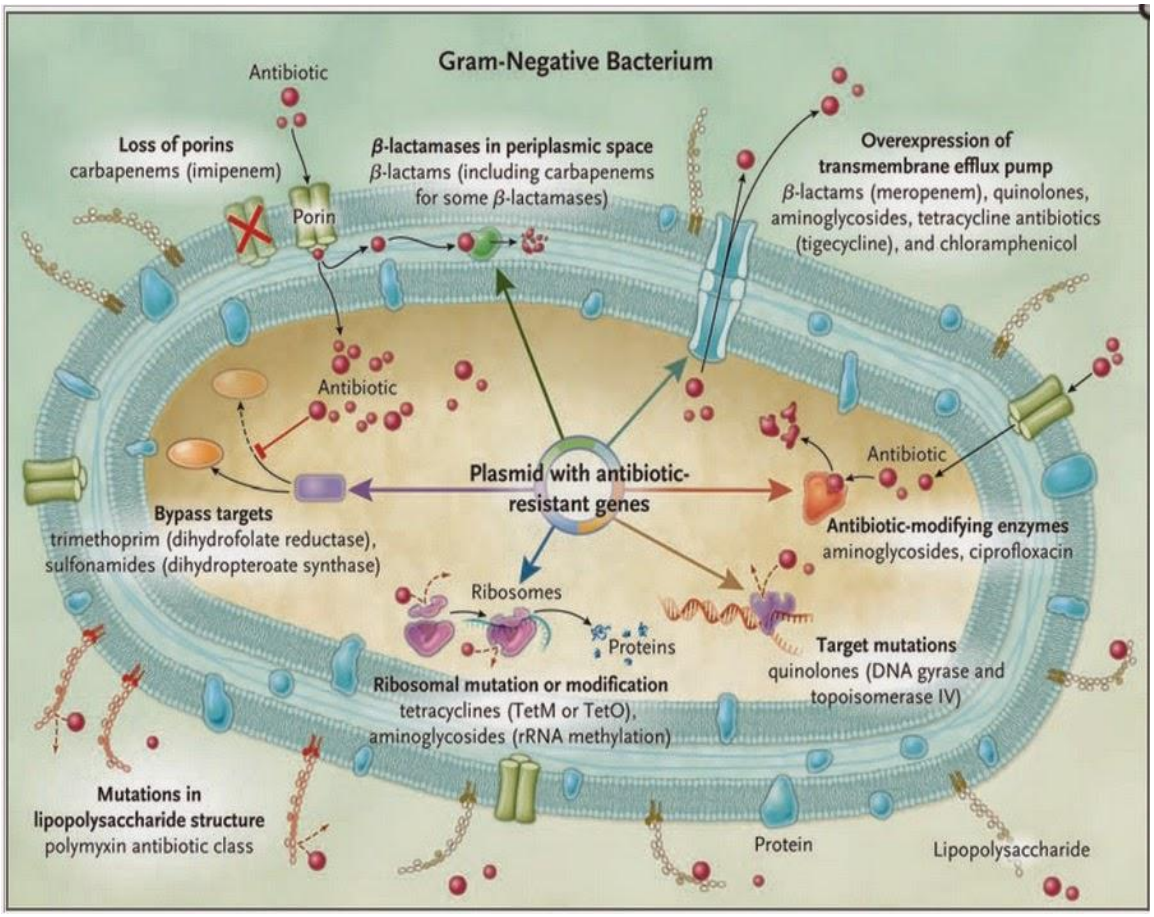


Figure 5.mechanisms of β-lactam resistance in Gram-negative bacilli.

β-lactamases

Increased use of β-lactam antibiotics has been associated with the emergence of β-lactamase-mediated bacterial resistance. β-lactamases are enzymes that inactivate β-lactam antibiotics by cleaving the β-lactam ring through an irreversible hydroxylation of an amide bond (Figure 6).Two classification schemes for β-

lactamases are currently in use, the functional (Bush-Jacoby group) and molecular classification (Ambler classification).Functional classification aligns β-lactamases based on their ability to hydrolyze specific β-lactams classes and on the inactivation properties of β–lactamase inhibitors. The widely used molecular classification (Ambler classification) is based on the amino acid sequences and divides the β-lactamases into four classes. Class A, C, and D enzymes require serine at their active sites for substrate hydrolysis, whereas class B metallo β-lactamases utilize divalent zinc ion for substrate hydrolysis. The production of β-lactamases may be inducible or constitutive. In Gram-positive bacteria, β-lactamases are generally inducible, resulting in larger amounts of enzymes produced in the presence of an antibiotic. In Gram-negative bacteria, the production of β-lactamases is frequently constitutive, i.e. the enzyme is produced even when the antibiotic is not present. However, ampicillin class C β-lactamases (AmpCs) are inducible in certain Gram-negative bacteria, such as *Escherichia*, *Enterobacter*, *Serratia* and *Citrobacter* species.[70,71] Transmissible plasmids have acquired genes for AmpC enzymes, which subsequently can now appear in bacteria deficient or poorly expressing a chromosomal AmpC gene, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis.*[119]

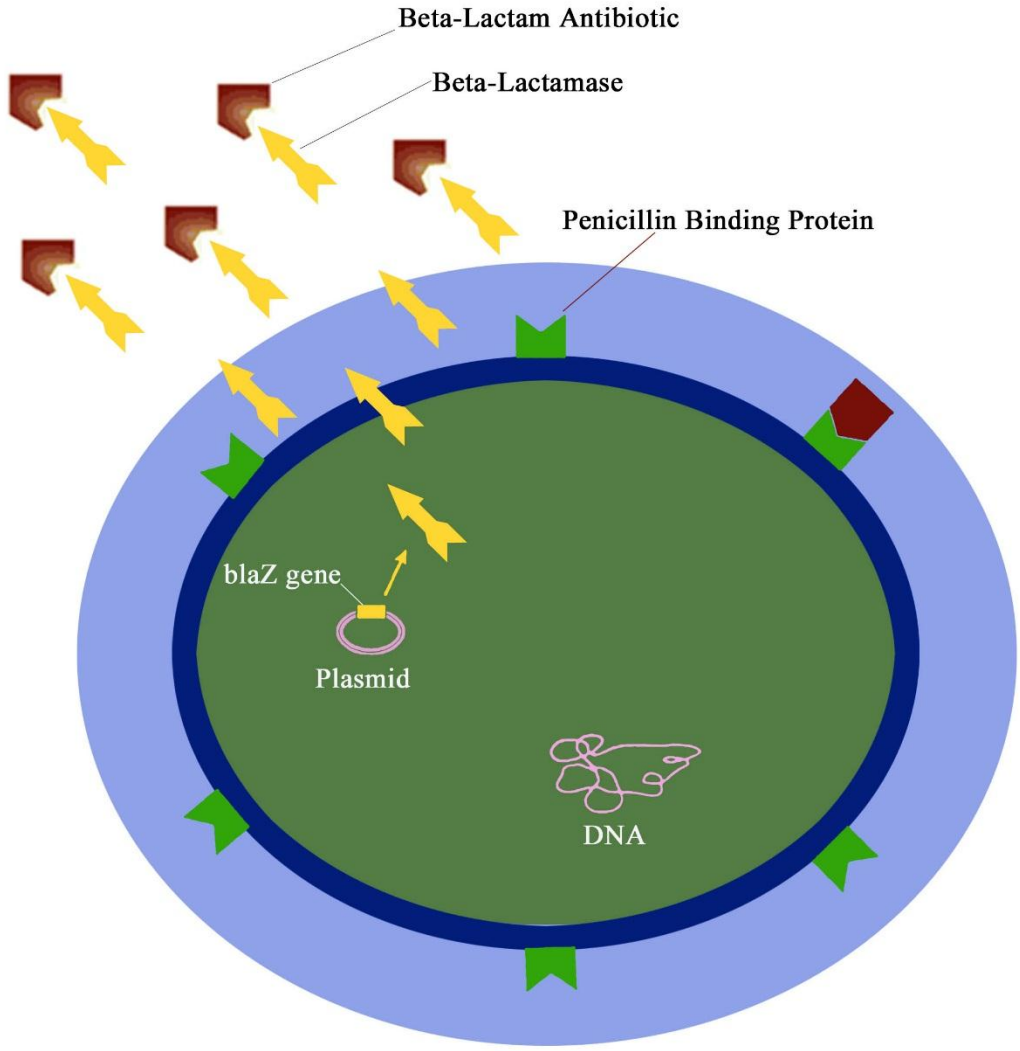


Figure 6. Diagram showing the mechanism of action of β-lactamases.

β-lactamase enzyme causes an opening in the β-lactam ring represented an active penicillin ineffective (inactive penicillin). After the introduction of penicillin, resistance to this antibiotic emerged in *Staphylococcus aureus* due to a plasmid mediated penicillinase that specifically hydrolyzed penicillin.[46,47] Penicillinase spread quickly to other species of staphylococci. Resistance to

penicillin led to the development of many new β-lactams; however, with each new class of β-lactams developed, new β-lactamases active against that class of antibiotics emerged. Some of these new classes of antibiotics were the 3rd and 4th generation cephalosporins and carbapenems which became the most common agents for treating infections caused by Gram-negative bacilli.[13,32,33] However, the rapid emergence of resistance to β-lactams due to AmpCs and extended-spectrum β-lactamases (ESBLs)is of a great public health alarm.[13,34] AmpCs are β–lactamases that hydrolyze broad and extended-spectrum cephalosporins, but are not inhibited by β-lactamase inhibitors.

Evolution of β-lactamases

Since the discovery of plasmid (R factor) mediated antibiotic resistance in the1940s, the scientific community has observed different strategies embraced by the bacteria to acquire and distribute antibiotic resistance. The b*la*-SHV type ESBLs have initiated from the chromosomal *bla*-SHV of *K. pneumoniae*. The fact that first *bla* gene in *Klebsiella spp* was found on a transposon suggests that this mobile genetic element has played a role in the escape of genes from the chromosome into the plasmid[85]. The origin of *bla*-TEM enzymes is less certain, but they are almost always found encoded on plasmids with exceptions of *bla*-TEM-12 and *bla*-TEM-134, whose genes were found on chromosomes [86]. The origin of *bla*-CTX-M genes have drawn to the chromosomal *bla* genes of *Klebsiella spp*. Phylogenetic studies

suggest that *bla*-CTX-M genes of *bla*-CTX-M-1 cluster has evolved from chromosomal *bla* genes of *K.cryocrescens*, *bla*-CTX-M-2 cluster from K. *ascorbata* whereas CTX-M-8, CTX-M-9 andCTX-M-25 clusters have evolved from *K georgiana* [90]. Mobile genetic elements such as transposon (Tn*1*, Tn*2*, Tn*3*, Tn*21*), integrons (Class 1, 2), insertion sequences (IS*Ecp1*, IS*26*, IS*CR1*) and even bacteriophages are believed to have helped these genes cross the genus barrier [88]. While mutations are the primary reason for the large numbers of allelic variants, recombination events too have played a role [90].

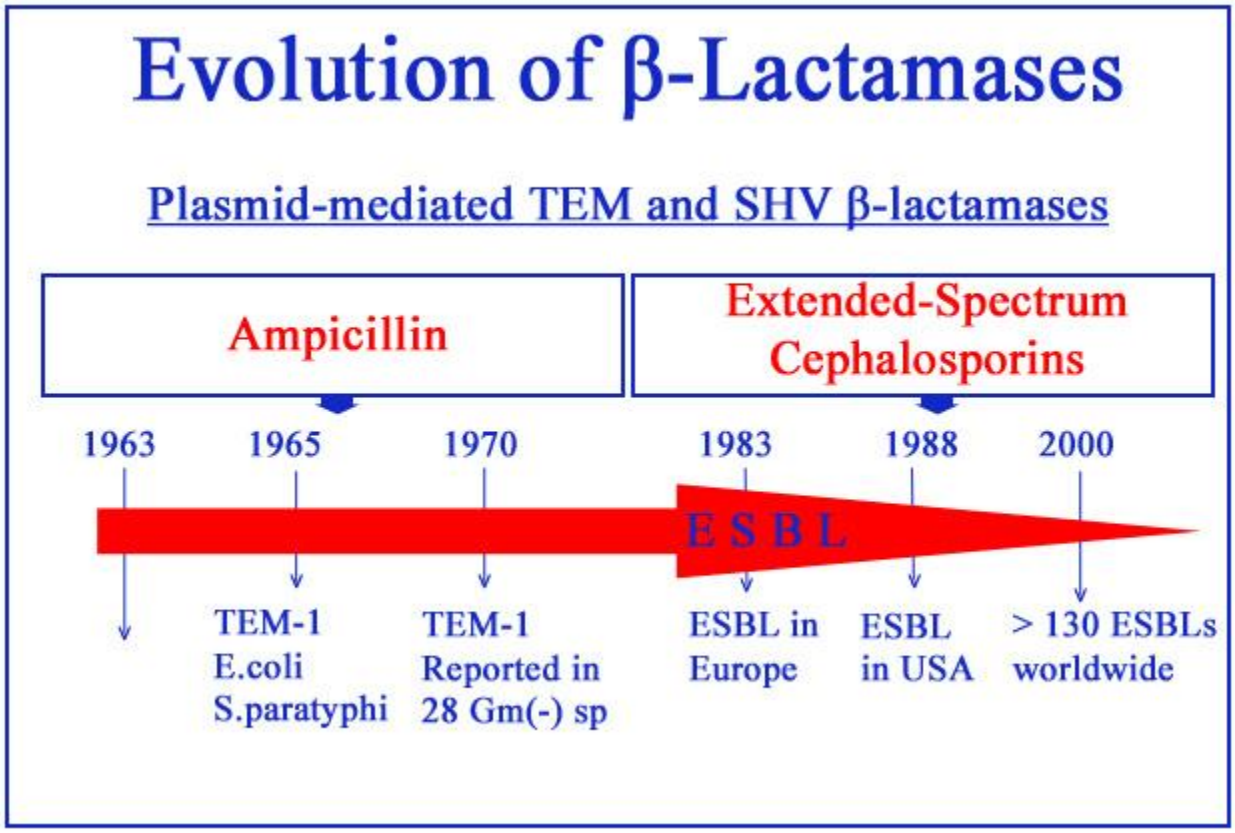


Figure 7: Evolution of β-lactamases

Extended spectrum *β* -lactamase-producing bacteria

Extended spectrum β-lactamases are β-lactamase enzymes that have the ability to hydrolyze penicillins, extended-spectrum cephalosporins (oxyimino-β-lactams), and aztreonam, but not carbapenem or cephamycin antibiotics. ESBLs are reported worldwide among different bacterial species, including Enterobacteriaceae and non-fermentative Gram-negative bacteria such as *Pseudomonas* and *Acinetobacter* species.[72]

Class A extended-spectrum β –lactamases

bla-CTX-M (cefotaximase, Munich), *bla*-SHV (sulfhydryl variable) and *bla*-TEM (Temoneira) types are the most clinically common Ambler class A ESBL enzymes, and susceptible to β-lactamase inhibitors (Table 1).[72] The first plasmid-encoded ESBL enzymes were identified among Enterobacteriaceae in the 1980s; they were derived mainly from the narrow spectrum enzymes (*bla*-TEM-1, *bla*-TEM-2, or *bla*-SHV-1) by point mutations resulting in amino acid changes conferring an extended-spectrum of activity against β-lactams. Unlike *bla*-SHV and *bla*-TEM enzymes, *bla*-CTX-M enzymes had independent evolution. Although ESBLs were first reported as plasmid-encoded, they are not completely plasmid-encoded; recent studies have shown that ESBL genes are also carried on chromosomes.[69]

bla-CTX-M type ESBLs

bla-CTX-M enzymes were derived from the chromosomal cephalosporinase of *Kluyvera* species, and the first *bla*-CTX-M enzyme was discovered in an *E. coli* clinical isolate from Munich, Germany in 1989.It was suggested that *bla*-CTX-M genes was apprehended by mobile elements from the chromosomes of *Kluyvera* species. *bla*- CTX-M enzymes are classified into five groups; *bla*-CTX-M-1, -2, -8, -9 and 25. Enzymes within the same *bla*-CTX-M group share about 94% similarity, however ≤90% similarity is noticed between members of different groups. *bla*-CTX-M enzymes have greater hydrolytic activity against cefotaxime than other oxyimino-β-lactams, hence the name *bla*-CTX-M (cefotaximase, Munich). However, some *bla*-CTX-M enzymes have greater activity against ceftazidime. *bla*-CTX-M-15, -16, -19, -25, -27, and -32 have higher hydrolytic activity against ceftazidime due to point mutations around their active sites.[74] In addition, *bla*-CTX-M enzymes exhibit higher significant hydrolytic activities against cefepime than detected with other ESBL types. All *bla*-CTX-M enzymes have ESBL phenotype, and are commonly identified among Enterobacteriaceae, mainly *Esch. coli* and *K. pneumoniae*.[73]

bla- TEM type ESBLs

The first *bla*-TEM enzyme (*bla*-TEM-1) was discovered in 1965 in Athens (Greece) from a patient infected with *Esch. coli*, named Temoneira, hence the name *bla*-TEM. *bla*- TEM-1 is the common plasmid-mediated β-lactamase causing ampicillin resistance among Enterobacteriaceae; responsible for up to 90% of ampicillin resistance in *Esch. coli*. Both *bla*-TEM-1 and *bla*-TEM-2 have hydrolytic activity against ampicillin and early-generation cephalosporins. All *bla*-TEM-type ESBLs were derived from *bla*-TEM-1 and *bla*-TEM-2.*bla*-TEM-type ESBLs hydrolyze ceftazidime with higher efficiency than other oxyimino β-lactams. Some *bla*-TEM-type ESBL variants possess silent substitutions without amino acid changes (*bla*-TEM-1a and *bla*-TEM-1b) and others have single amino acid changes enough to assign a new number (*bla*-TEM-3, -4, -5, etc.). Up to date, about 84 *bla*-TEM variants have an ESBL phenotype. These are commonly identified in Enterobacteriaceae.[69]

bla-SHV type ESBLs

bla-SHV type ESBLs are derived from narrow-spectrum *bla*-SHV-1 or *bla*-SHV-11 β-lactamases as a result of point mutations. Initially, *bla*-SHV-1 was described as plasmid-encoded and later as chromosomally encoded among *K. pneumoniae*

isolates. *bla*-SHV-1 or *bla*-SHV-11 is responsible for ampicillin resistance and accounts for up to 80% to 90% of resistance in *K. pneumoniae*. *bla*-SHV type ESBLs have greater hydrolytic activity against ceftazidime than other oxyimino-β-lactams (cefotaxime, cefepime). *bla*-SHV type ESBLs are mainly found in Enterobacteriaceae and *P. aeruginosa* and the most common are *bla*-SHV-2a, -5 and -12.[75]

Class D extended-spectrum β–lactamases

bla-OXA type ESBLs are the only Ambler class D ESBL enzymes. *bla*-OXA type ESBLs are less common and were named *bla*-OXA for their greater activity against oxacillin or cloxacillin and extended-spectrum β-lactams. *bla*-OXA type ESBLs primarily hydrolyze ceftazidime and are poorly inhibited by clavulanic acid. About 16 *bla*-OXA type ESBL enzymes have been discovered; especially in *Pseudomonas aeruginosa* and *Esch. coli* strains.[69]

Global prevalence of ESBL-producing bacteria

Prevalence of ESBL-producers

Enterobacteriaceae are the main bacterial species associated with the production of ESBLs.[38] Enterobacteriaceae are commonly found in human

gastrointestinal tract (GIT). However, these organisms can cause a variety of extra-intestinal infections in the lower and upper urinary tracts, bloodstreams, central nervous system, pelvis or abdomen and wounds. The most important Enterobacteriaceae species associated with ESBL production are *Esch. coli* and *K. pneumoniae*.[39] ESBL-producing Enterobacteriaceae (ESBL-PE) often display resistance to multiple drugs and co-resistance to other antibiotics used for treatment (aminoglycosides, fluoroquinolones and trimethoprim) is common. Treatment options for severely ill patients are limited, resulting in increasing morbidity and mortality rates. ESBL- producing Enterobacteriaceae species are therefore a growing public health worry worldwide. The prevalence and distribution of ESBLs varies widely in different geographical regions, differs from country to country, as well as within the country between hospital and community settings.[38,39]

Table 1. Classification schemes for bacterial β-lactamases, modified from Bush and Jacoby 2010.

Ambler Class	Bush group	Representative examples	Gene location	Substrate preference	Inhibited by
A	2a,2b	TEM-1/2 SHV variants	Plasmid, Plasmid / chromosome	Penicillin	Clavulanate, sulbactam
	2be	ESBL (TEM-3-TEM-100, CTX-M) SHV-3-SHV-54	Plasmid Transposon Plasmid/chromosome	Ampicillin, 2nd and 3rdgeneration cephalosporins	Clavulanate, tazobactam
	2br	TEM (IRTs) SHV IRTs	Plasmid, Plasmid / chromosome	Ampicillin, Cephalosporins	Tazobactam
	2c	Carbenicillin Hydrolyzing		Carbenicillin	
	2e	Cephalosporinase		Cephalosporins	Clavulanate ,Tazobactam
	2f	NMC-A, IMI, SME-1 to SME-3	Chromosome	Ampicillin, aztreonam, carbapenems	Clavulanate ,Tazobactam
	2f	KPC-1 to KPC-3, GES-1 to GES-2	Plasmid Plasmid-integron	Ampicillin, cephalosporins, aztreonam, carbapenems	Clavulanate , Tazobactam
B	3a,3b,3c	Zinc metallo β – lactamases IMP-1 to IMP-14, VIM-1 to VIM-3	Some chromosome, often plasmid and integrin	Cephalosporins, cephamycins, penicillins	EDTA
C	1	AmpC-type CMY, MOX, FOX, ACT-1 DHA-2	Originally only chromosome, now plasmids	Cephalosporins, cephamycins, penicillins	Cloxacillin
D	2d	OXAs, PSEs, OXA-23-27, 40, 48	Chromosome, plasmids some on integrons	Oxacillin, Penicillin, carbenicillin, cephaloridine plus carbapenems	Clavulanate
	4	Miscellaneous AVS-1			

EDTA: Ethylene diamine tetra acetic acid

The Bush and Jacoby group or functional classification takes into account the substrate and inhibitor profiles whereas the Ambler or molecular classification is by

protein sequence, whereby the beta-lactamases are classified into four molecular classes (A,B, C and D) based on conserved and distinguishing amino acids motifs.(CA, clavulanic acid; TZB, tazobactam; EDTA, ethylene diamine tetra acetic acid)

Prevalence of ESBL-producing bacteria in Europe

In Europe, the prevalence of ESBL- producing Enterobacteriaceae varies between countries.[40]The prevalence is low in Scandinavian countries but is rapidly increasing. A surveillance study conducted in South Western Sweden during 2004 to 2008 reported a greater increase in the prevalence of *bla*-CTX-M-producing *Esch. coli* isolates in hospitals (0.2% - 2.5%) than in the community (0.2% – 1.6%). In contrast, the prevalence of ESBL- producing Enterobacteriaceae is high in the Southern and Eastern parts of Europe.[40] Spain study shoes 7 (6.7%) of 105 healthy humans were carriers of ESBL-producing *Esch. coli,* and *bla*-CTX-M-14 was the most dominantenzyme.[76] A study conducted in Portugal, the faecal carriage of ESBL-producing *Esch. coli* in patients with UTI caused by these microorganisms was 68%(36/53), but the domestic members and the non-domestic relatives of these patients also had ESBL-producing *Esch. coli* in faecal samples, 27.4% (20/73) and 15.6% (5/32),respectively, whilst in healthy unrelated controls the faecal carriage rate was 7.4%(4/54).[77]In Germany, ESBL-producers were recovered from animals, including dogs, cats and horse, and the authors also described an active transmission of such organisms between humans and animals.[78]

Prevalence of ESBLs in America

In America, data on ESBL- producing Enterobacteriaceae organisms both in hospital and community settings are scarce. As well, a recent survey from the USA including 26 hospitals from 20 states reported the prevalence of 6.4% (195/3049) of ESBL-producers among Enterobacteriaceae isolates.[79]

Prevalence of ESBLs in Africa

In Madagascar, ESBL- producing Enterobacteriaceae carriage was described in 10.1% of 484 patients of all ages (median age, 28 years) attending a health care setting for the first time. As in other studies, *bla*-CTX-M-15 was frequently identified, 86.8% of ESBL- producing Enterobacteriaceae isolates, mainly in *Esch. coli* and *K. pneumoniae* isolates. Other ESBL- producing Enterobacteriaceae isolates identified *bla*-SHV-12-producing *Ent. cloacae*, *bla*-CTX-M-3-producing *Esch. coli*, *bla*-CTX-M-1-producing *Esch.coli* and *bla*-SHV-2a-producing *K. pneumoniae*. In the latter study, the occupational status of the head of domestic and shortage were risk factors for ESBL- producing Enterobacteriaceae carriage.[20]

Prevalence of ESBLs in Asia

A cross sectional study conducted in rural community of Thailand reported a very high ESBL- producing Enterobacteriaceae faecal carriage of 58.2% in 160 healthy adult individuals (mean age; 56.0 ± 9.8 years).[80]InChina, among 270

healthy adults, the faecal carriage rate of ESBL-producing *Esch. coli* was7%. Unlike in most parts of the world where *bla*-CTX-M-15 is predominant, in South-East Asia the most common ESBL enzyme is *bla*-CTX-M 14.[81]

Prevalence of ESBL producers in India

The data was collected from international surveillance studies, which included multiple centers from India. As a part of the MYSTIC Surveillance program, bacterial samples were collected from six cities (New Delhi, Lucknow, Indore, Mumbai, Bangalore and Vellore) across India prior to 2000. The prevalence of ESBL producers in that study was very high; 92% of Esch. coli and 96% of K. pneumonia were found to be ESBL producers.[91] Interestingly, another publication of the same study reported ESBL production in >61% and >55% among Esch. coli and Klebsiella spp, respectively.[92]India is included in the Asia-Pacific SMART study in 2007 and samples collected from nine centers in that period showed prevalence of ESBLs in 79% of Esch.coli and 69.4% of K. pneumoniae isolates [93]. In one of the centers included in that study, a prevalence rate of 94.1% was detected among Esch. coli isolates. ESBL producers were found responsible for 78.9% of both nosocomial and community acquired infections in that study. In the 2008 Asia-Pacific SMART study, samples collected from seven centers across India described a prevalence of ESBLs in 61.2% of Esch. coli and 46.8% K.pneumoniae isolates.[94] The prevalence rates increased marginally in the subsequent study. The 2009 Asia-

Pacific SMART study, reported a prevalence rate of 67.1%among Esch. coli and 56.8% among K. pneumoniae isolates.[95]

In the same period, multinational Asian Network for Surveillance of Resistant Pathogens (ANSORP) surveillance study reported similar prevalence (57.1%) of ESBL-Klebsiella from Indian centers.[96]

The regional Resistance Surveillance (RRS) program in the Asia-Pacific (APAC) region reported a slightly increased prevalence of ESBLs; 78% of *Esch. coli* and 64% of *K. pneumoniae* isolates were found to be ESBL producers.[97]Although there are numerous studies from various parts of India, they differ widely in patient population, sample type and size, detection methodology and the study period. Hence, even with a vast amount of data, meaningful interpretation is difficult to achieve.

In the period 2013-14, the rates of ESBL detection among *Esch. coli* ranged from24-90% and that of *K. pneumoniae* ranged from 9-80% in the Northern parts of India.[98]

In the Eastern parts, the rates ranged from 12-89% among *Esch. coli* and 26-93% among *K. pneumoniae* isolates.[100]

In the central parts of India, the rates ranged from 41-50% among *Esch. coli* and 26-48% among *K. pneumoniae* isolates.[101]

In the western India, the rates ranged from 20-62% among *Esch. coli* and 15-67%among *K. pneumoniae* isolates.[117]

In the southern India, the rates ranged from18-73% among *Esch. coli* and 33-63% among *K. pneumoniae* isolates.[102]

ESBL types in India

- The first report of an ESBL producer from India came in 1998, when *bla*-SHV-5 ESBL was discovered among *Salmonella* isolated from patients during an outbreak in a burns ward at Delhi.[103]
- In 2001, Karim et al reported detection of 70 *bla*-CTX-M-15 in clinical isolates of *Esch. coli, K. pneumoniae,* and *Ent. aerogenes* collected in 1999 in a hospital at Delhi.[112] This is the first account of a *bla*-CTX-M enzyme that was also capable of hydrolyzing ceftazidime.
- In 2006, Ensor et al reported detection of *bla*-CTX-M-15 in 73% of *Esch. coli* and *K.pneumoniae* isolates collected from centers located in Aligarh, Varanasi and Hubli.[104]
- In response to this publication, Walsh et al reported that their study team, which had collected samples from New Delhi, Mumbai, Indore, Lucknow, Bangalore and Vellore prior to 2000, had identified *bla*-CTX-M- 15 in 83% of *Esch. coli* and 75% of *Klebsiella sps.*[91]
- *bla*-CTX-M -15 still continues to be the dominant *bla*-CTX-M type in India. Several studies from India have detected *bla*-CTX-M genes among the

clinical isolates by amplifying only a part of the *bla*-CTX-M gene. Many of these studies had used multiplex PCR protocols, which allow the researchers to detect only the *bla*-CTX-M groups but not their individual members.

- In 2008, a study from Chennai reported detection of *bla*-CTX-M-1 ESBL among Enterobacteriaceae members.[105] Authors of that study, used the same primer pairs for another study and reported detection of *bla*-CTX-M-28,*bla*-CTX-M-1, *bla*-CTX-M-15 and *bla*-CTX-M-28 belong to the *bla*-CTX-M-1 cluster; *bla*-CTX-M-15 differs from *bla*-CTX-M-1 by four amino acids but *bla*-CTX-M-28 differs from *bla*-CTX-M-15 by one amino acid at position289. The primers used by the authors of those two studies are more suitable for detecting *bla*-CTX-M-15 ESBLs than *bla*-CTX-M-1 or *bla*-CTX-M-28. The possibility of miss identification of *bla*-CTX-M-28 by using primers designed for *bla*-CTX-M-15 pointed out by Menezes et al.[106]
- In 2009, a report from Chennai detected *bla*-SHV-28 in a clinical isolate of *K.pneumoniae* and claimed it to be an ESBL since the isolate gave positive ESBL phenotypic test and is electric focusing revealed only a single band .As now, *bla*-SHV-28 is not established an ESBL. Among the few reports on *bla*-SHV type ESBLs, *bla*-SHV-12, followed by *bla*-SHV-2, *bla*-SHV-4 and *bla*-SHV-5 are the only ones reported from India.[99]

Detection methods for ESBL- and carbapenemase-producers

Detection of extended-spectrum β -lactamases

Extended-spectrum β -lactamases can be detected with phenotypic or genotypic approaches.

Phenotypic tests

The phenotypic detection of ESBLs includes screening and confirmatory steps. Screening involves testing for reduced susceptibility to extended spectrum β-lactams, including cefotaxime, ceftazidime, cefpodoxime, ceftriaxone or aztreonam. Cefpodoxime is the most sensitive indicator for detecting ESBLs, because it can be hydrolyzed by all common *bla*-CTX-M, *bla*-SHV, and *bla*-TEM ESBLs. But, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime. Confirmatory phenotypic tests are based on the demonstration of synergy between the Extended spectrum β-lactams and β-lactamase inhibitors particularly clavulanic acid. Several confirmatory tests including combination disc test, double-disc diffusion test, ESBL E-test or ESBL NDP test are used in different settings. In addition, auto mated systems (the Vitek ESBL and BD Phoenix ESBL tests) that

use the above mentioned detection principle are in use in many clinical microbiology laboratories.[82,83]

Combination disc test (CDT)

Principle:

The CDT detects the production of ESBL enzymes based on that they hydrolyze cephalosporin antibiotics and are inhibited by clavulanic acid (Figure8).

Test:

Ceftazidim and Cefotaxime or Ceftriaxone alone and in combination with clavulanic acid is preferred in isolates with inducible AmpC enzymes as this antibiotic is stable to AmpC β-lactamases.[84] AmpC enzymes interfere with clavulanic acid synergy and detecting ESBL production in such isolates using cefotaxime or ceftazidime is challenging.

Advantage:

The test is cheap and easy to perform, and interpretation is straight forward.

Sensitivity and specificity:

The sensitivity and specificity of this test using cefotaxime and ceftazidime alone and in combination with clavulanic acid have been reported to be 96% and 100%, respectively; however, the sensitivity of this test can be further increased by using cefepime alone and in combination with clavulanic acid.[84]

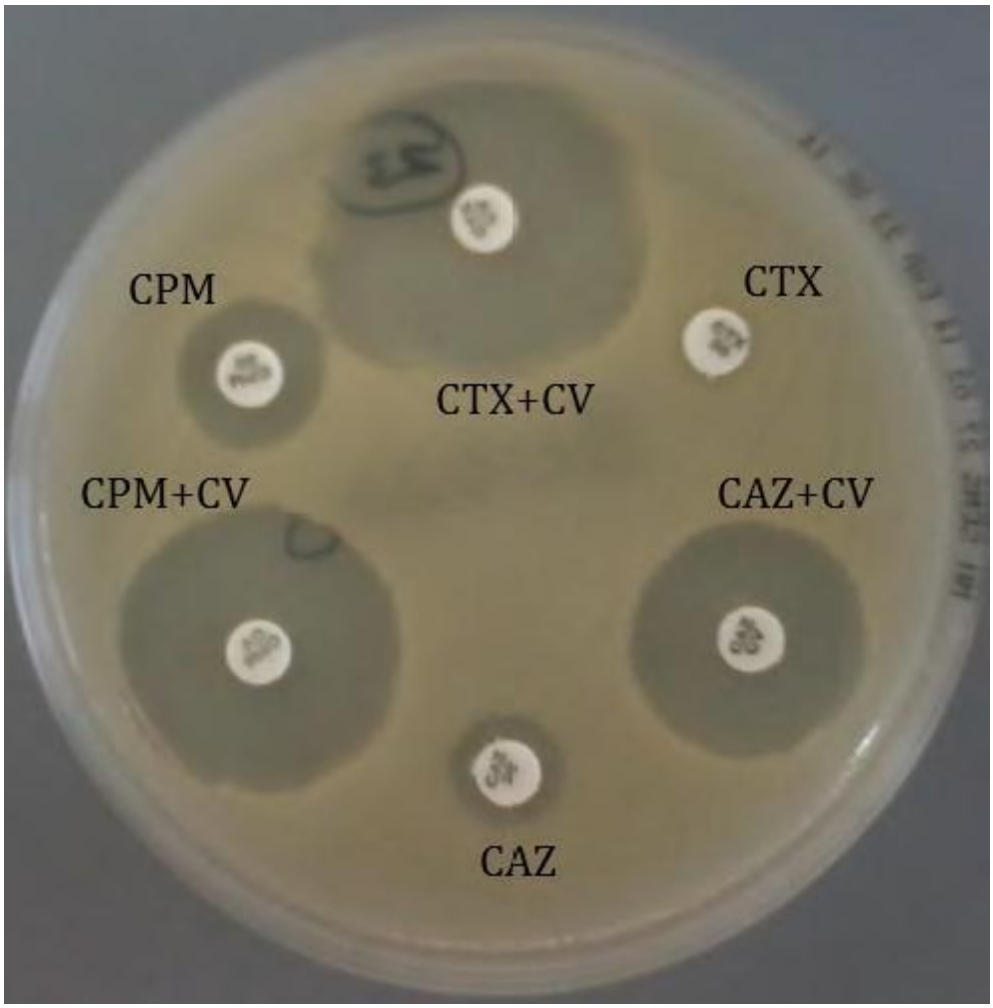


Figure 8. Combination disc test (CDT) showing an increase by at least 5mm for cefotaxime-clavulanic acid, ceftazidime-clavulanic acid and cefepime-clavulanic acid as compared to cefotaxime, ceftazidime and cefepime, respectively. CPM, Cefepime; CPM+CV, Cefepime-clavulanic acid; CAZ, Ceftazidime; CAZ+CV, Ceftazidime-clavulanic acid; CTX, cefotaxime; CTX+CV, Cefotaxime-clavulanic acid.

Double-disc Diffusion test (DDT)

- The DDT was the first test designed to detect ESBL production in Enterobacteriaceae.
- The cephalosporin discs are applied to a plate next to a disc containing clavulanate (amoxicillin-clavulanate).

- A positive result is indicated when the zone of inhibition around any of the cephalosporin discs is augmented in the direction of the disc containing clavulanate.
- This method has remained a reliable test for the detection of ESBLs in clinical microbiology laboratories.
- In addition, the DDT is also cheap and easy to perform; however, reading the results is some time difficult.[83]

ESBL E-tests

- The ESBL E-tests contain the gradients of cephalosporin alone at one end of the strip and combined with 4 μg/ml of clavulanic acid on the other end.
- The ESBL E-test is considered positive when the minimum inhibitory concentration (MIC) of the cephalosporin combined with clavulanic acid is reduced by ≥8-fold as compared with the MIC of the cephalosporin alone or if a deformed ellipse or phantom zone is present.
- The ESBL E-test is easy to perform but the interpretation of results is sometimes challenging.
- In 2002, a study conducted in The Netherlands reported failure of laboratory technicians to recognize the phantom zones or ellipse deformation in 30% of cases.[83]

ESBL Nordmann Dortet Poirel (ESBL NDP) test

This is a newly established phenotypic test for the detection of ESBLs in Enterobacteriaceae. The ESBL NDP test detects the production of ESBL based on the hydrolysis of cefotaxime. A red phenol solution is used as a pH indicator, and when a protein extract from the bacterium is mixed with red phenol solution containing cefotaxime, ESBLs contained within the protein extract hydrolyze the antibiotic, thus forming an acidic solution which causes the pH indicator to turn from red to yellow or orange. In the presence of tazobactam the activity of ESBLs will be inhibited, and the pH indicator will remain red (unchanged color), thus confirming the production of the ESBL (Figure 7).[47] Its sensitivity and specificity in detecting *bla*-CTX-M-type and *bla*-SHV-12 enzymes have been reported to be 98.0% and 99.8%, respectively.[47] Only few studies have evaluated this test.[47]

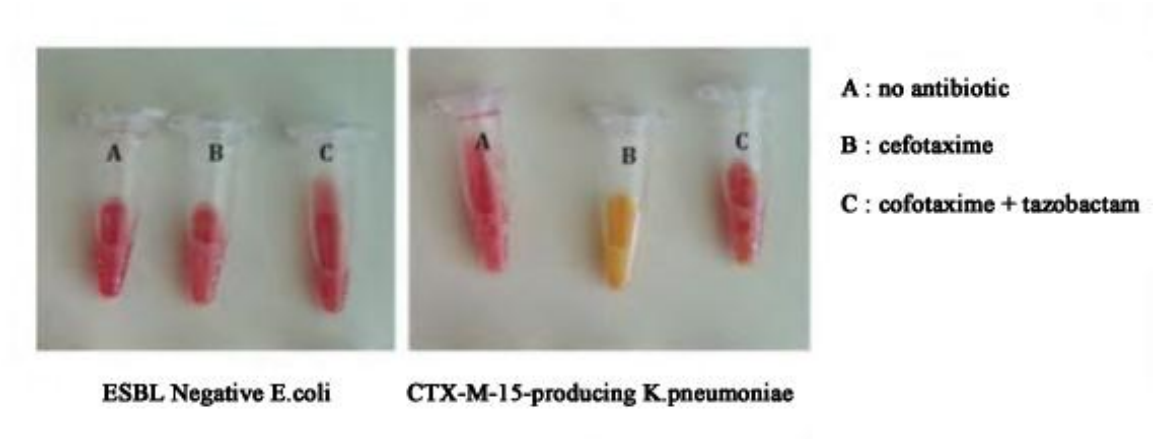


Figure 9. The ESBL NDP test showing the hydrolysis of cefotaxime in *bla*-CTX-M-15-producing *K. pneumonia* isolate (tube B). The action of ESBL enzyme (*bla*-CTX-M-15) is inhibited in the presence of tazobactam (tube C). There is no hydrolysis of cefotaxime in case of ESBL negative *Esch. coli* (tube B).

Genotypic tests

- Polymerase chain reaction (PCR) and subsequent sequencing of ESBL genes or DNA microarray-based methods are used for genotypic confirmation of the presence of specific ESBL genes. [39]
- Singleplex or multiplex PCR techniques may be used. Molecular tests remain the gold standard and are technically challenging, however, they have the advantage of identifying the specific type of ESBL gene present in a bacterium.[2]
- These techniques can detect low-levels of resistance, and can be performed without prior culture of the microbiological specimens.[2]
- The PCR tests are rapid and the results are usually obtained within six hours with excellent sensitivity and specificity. The main disadvantages of molecular techniques are their cost and inability to detect unidentified novel ESBL genes.

Conclusion:

The data on the prevalence of ESBL-producing bacteria in Indian setting continue inadequate. There are also few studies on the prevalence of ESBL-producers in healthy individuals in the community. In addition, only few studies describing the acquisition of ESBL- producing bacteria at birth have been conducted worldwide. Consequently, we sought to study the epidemiology of ESBL-producing bacteria in a birth cohort among apparently healthy South Indian, to describe community acquired ESBL producers and anti- microbial resistance.

Material and Methods

Material and Methods

Study design

Non randomized cross sectional study.

Place of study

Department of Microbiology Sree Mookambika Institute of Medical Sciences, Kulasekharam.

Period of study

The study was carried out from July 2015 to June 2016.

Study population

All clinical isolates of Esch.coli and Klebsiella, from urine, pus, blood, vaginal swab, ear swab, fluid aspirates(plural, acidic)and wound swab from the OPD and ward of Gynecology, Surgery, Medicine, Orthopedics, ENT,ICU and Nephrology departments.

Sample size

One hundred bacterial isolates.

Data collection

By excel sheet.

Sampling technique

Convenient sampling.

Data analysis

Descriptive analysis, Data entered in to Microsoft excel.

I. Significant level decided before starting of study: P= <0.05

II. Statistical tests to be used for data analysis: chi square test

III. Software(s) to be used for Statistical analysis: SPSS trail version 20.0

Sample size determination

$N = 4pq/d^2$	
n= Desired sample size, p= Prevalence of the disease / problem in community (50%) q= (1-p), d= Degree of accuracy (10%), 4= Confidence interval (95%)	$N= 4x50x50/(10)^2$ $= 4x50x50/10x10$ $= 4x50x50/100$ $= 100$ **Prevalence rate 50%**.

Table 2. Sample size determination

Collection of samples

Collection of urine sample

A sterile, dry, wide mouth container and request for 10-20 ml specimen. Clean catch, mid-stream urine. The female patients were advised to clean the area around the urethral opening with clean water, dry the area and collect the urine with the labia a part .The male patients were advised to wash the hands before collecting the specimen.[89]

Collection of skin wound, ear, fluids ect.

A sterile technique was applied to aspirate or collect pus or wound swab from abscess or wound infection, either by disposable syringe or by sterile swab stick.

Specimen was collected in a sterile container with cap, before an antiseptic dressing is applied. Special care was taken to avoid contamination with commensal organisms from the skin.[89]

Collection of blood

Blood was collected under strict aseptic precaution 5ml disposable syringe and immediately inoculated into BHIB and transfer to the microbiology lab and kept in to the incubator.[89]

Screening of ESBL-producing Esch.coli and Klebsiella

Inoculation of samples

All samples received for culture and sensitivity from various departments in Sree Mookambika Institute of Medical Science like urine, sputum, pus and blood, ect are processed. The urine samples were inoculated in CLED and BA, blood was inoculated in to BHIB and pus, sputum, swabs fluids and tips were inoculated in to BA and MA. These entire samples were processed immediately after receiving. Blood samples were sub cultured after overnight inoculation and on 5^{th} day in to the MA and BA for isolation. In between bottles are examined daily and sub-cultured into solid media if there is any visible sign of growth. After 24 hours period the plates were observed, the colony and organism was identified by biochemical test – Gram staining, motility, Oxidase, Catalase, mannitol motility, triple sugar iron, indole, citrate, and urease.[109]

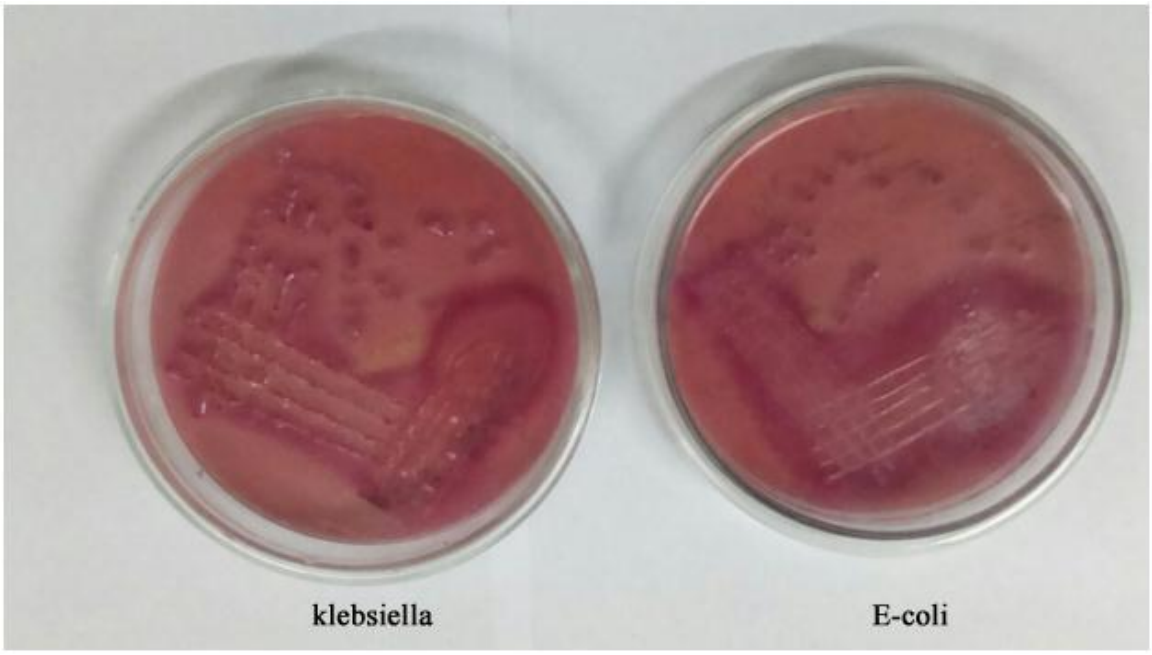


Figure 10: Culture identification

Isolation and identification of organisms

Grams staining

From the MA plate gram stain obtained to isolate Gram negative bacilli.

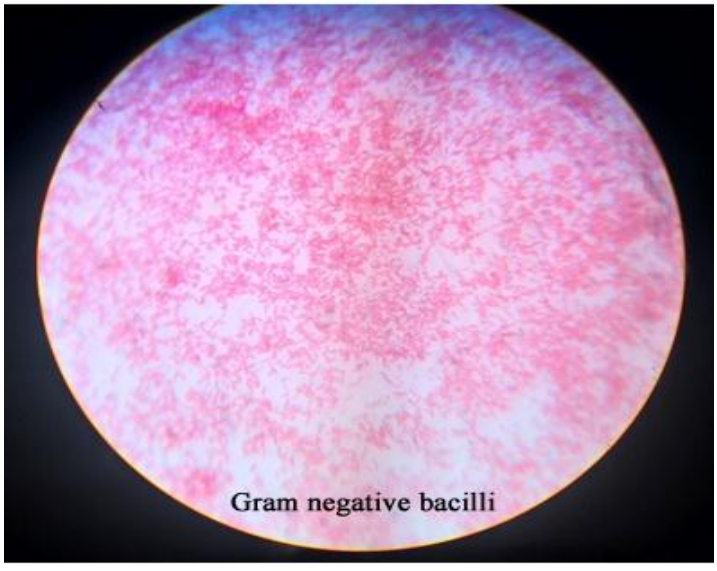


Figure 11: Gram stain microscope view

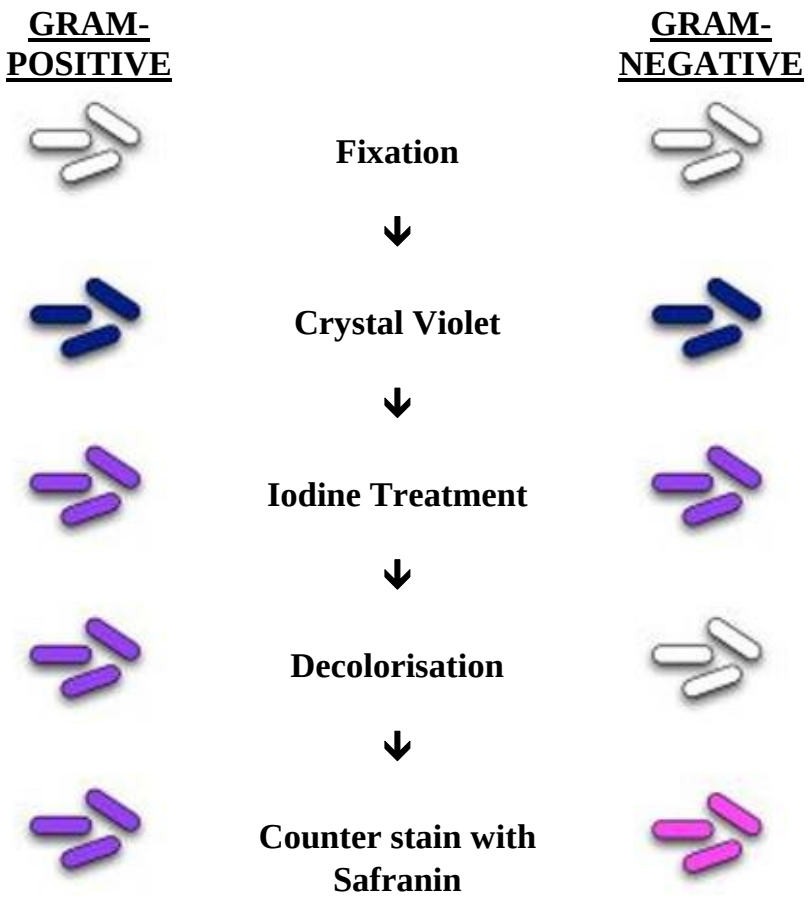


Figure 12: Procedure of Gram Staining[89]

Oxidase test:

This test was used to identify the organisms, which produce the enzyme oxidase.[89]

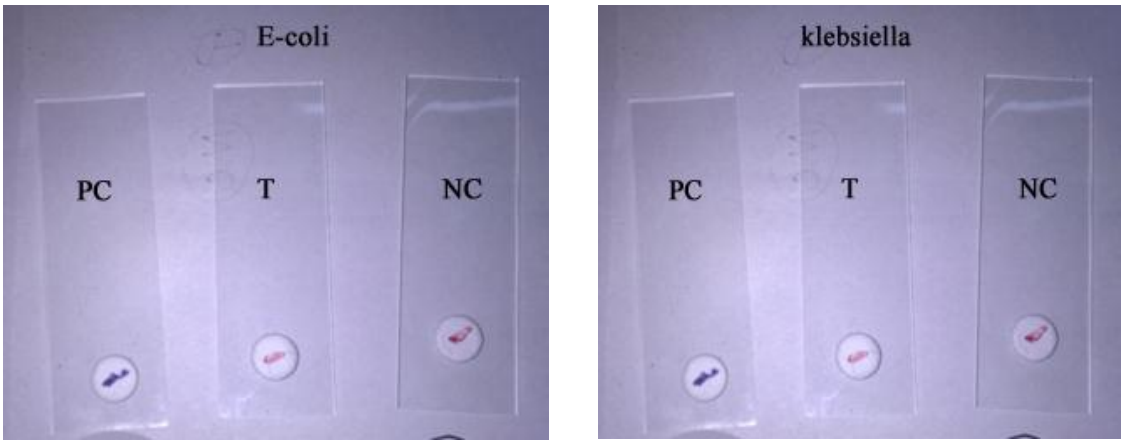


Figure 13: PC-Positive control, T-Test, NC-Negative control

Motility test:

Hanging drop used to test the motility of the bacteria. Here Esch.coli was motile and Klebsiella was non-motile.[89]

Figure 14: Manitol Motility test

Catalase test:

Tube catalase test used to identify the Enterobacteriaceae family. This study Esch.coli and Klebsiella were catalase positive.[89]

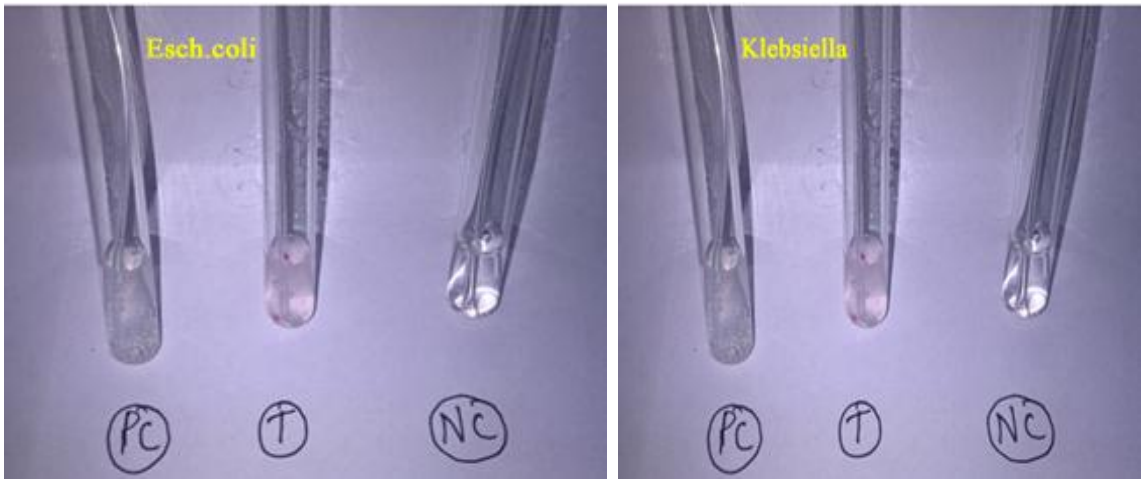


Figure 15: Catalase test (PC – Positive control, NC – Negative control, T - Test)

Citrate utilization test:

Simons citrate agar media was used for differentiating the intestinal bacteria and other micro organisms on the basis of citrate utilization. This study Esch.coli was not utilized citrate and Klebsiella utilized citrate.[89]

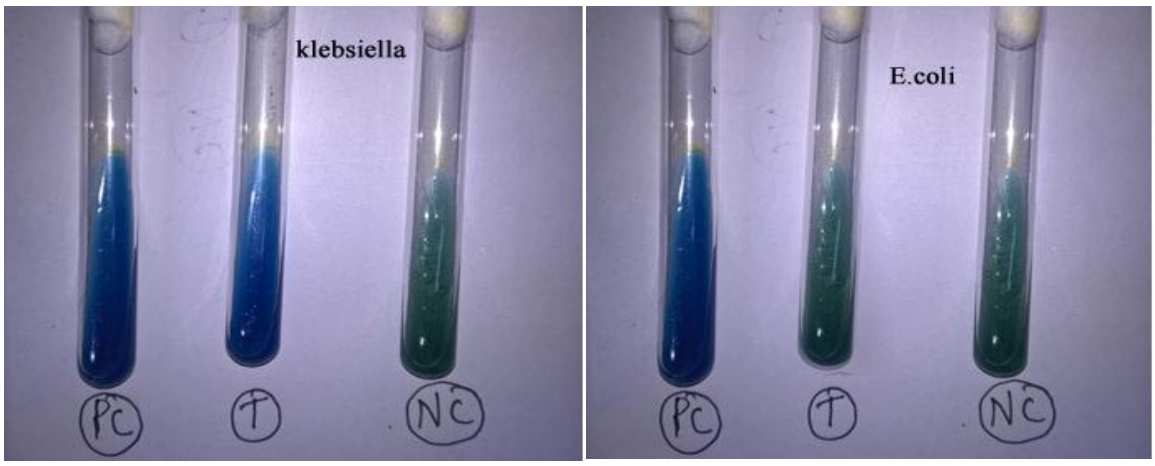


Figure 16: Citrate utilization test (PC-Positive control, T-Test, NC-Negative control)

Indole production test:

Indole production was tested for some bacteria, which has the ability to reduce tryptophan to indole. Indole production was detected by Kovacs reagent. Esch.coli and Klebsiella oxytoca are Indole positive and Klebsiella pneumonia are indole negative.[89]

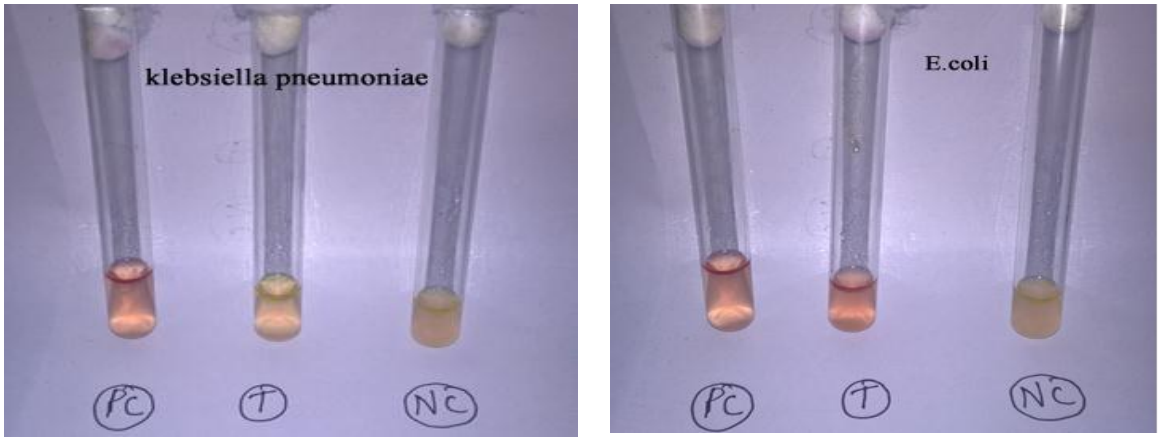


Figure 17: Indole production test. ***(***PC-Positive control, T-Test, NC-Negative control)

Triple sugar iron agar (TSI):

This media was used for initial identification of Gram negative bacilli, particularly members of *Enterobacteriaceae*. The three primary characteristics of a bacteria to be include in the ability to ferment carbohydrate (lactose, sucrose, glucose,), ability to produce gas, and the production of hydrogen sulfide gas. Esch.coli and Klebsiella fermented all the three carbohydrates and produced gas without H_2S.[89]

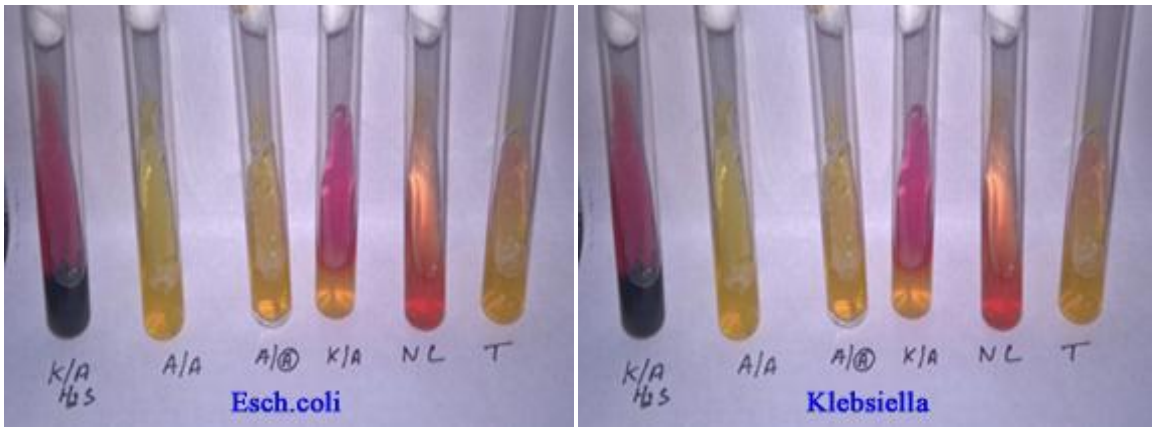


Figure 18: K/A H_2S- Alkaline/Acid with hydrogen sulfide gas, A/A-Acid/Acid, A/A –Acid/Acid with Gas, K/A-Alkaline/Acid, NC-Negative control, T-Test (Acid/Acid with Gas).

Manitol motility test:

Semisolid media used to see the motility and manital fermentation. Esch.coli and Klebsiella fermented manitol were else Klebsiella was non moile and Esch.coli was motile.[89]

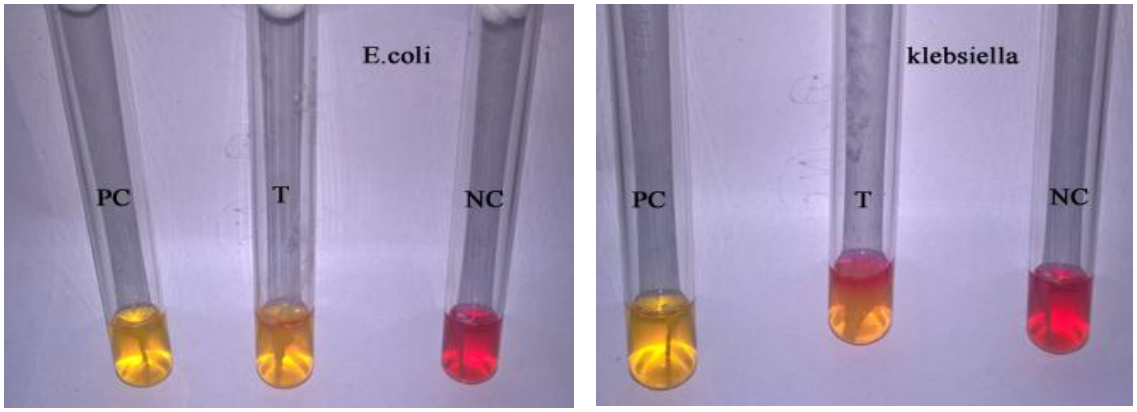


Figure 19: Manitol motility test **(**PC-Positive control, T-Test, NC-Negative control)

Urea hydrolysis test:

Christensen's urea medium used to hydrolysis of urea to produce ammonia and alkalinize the media. Esch.coli is not hydrolysing and Klebsiella was hydrolyzing urea.[89]

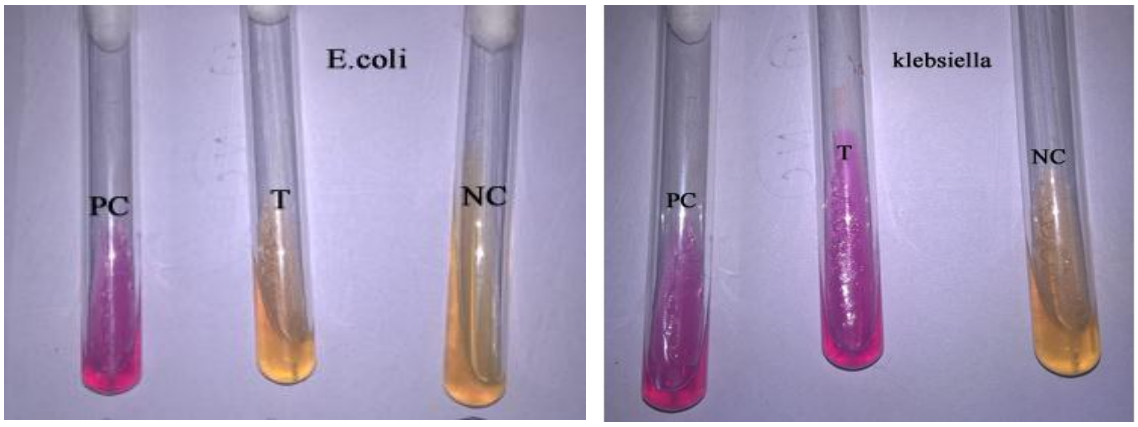
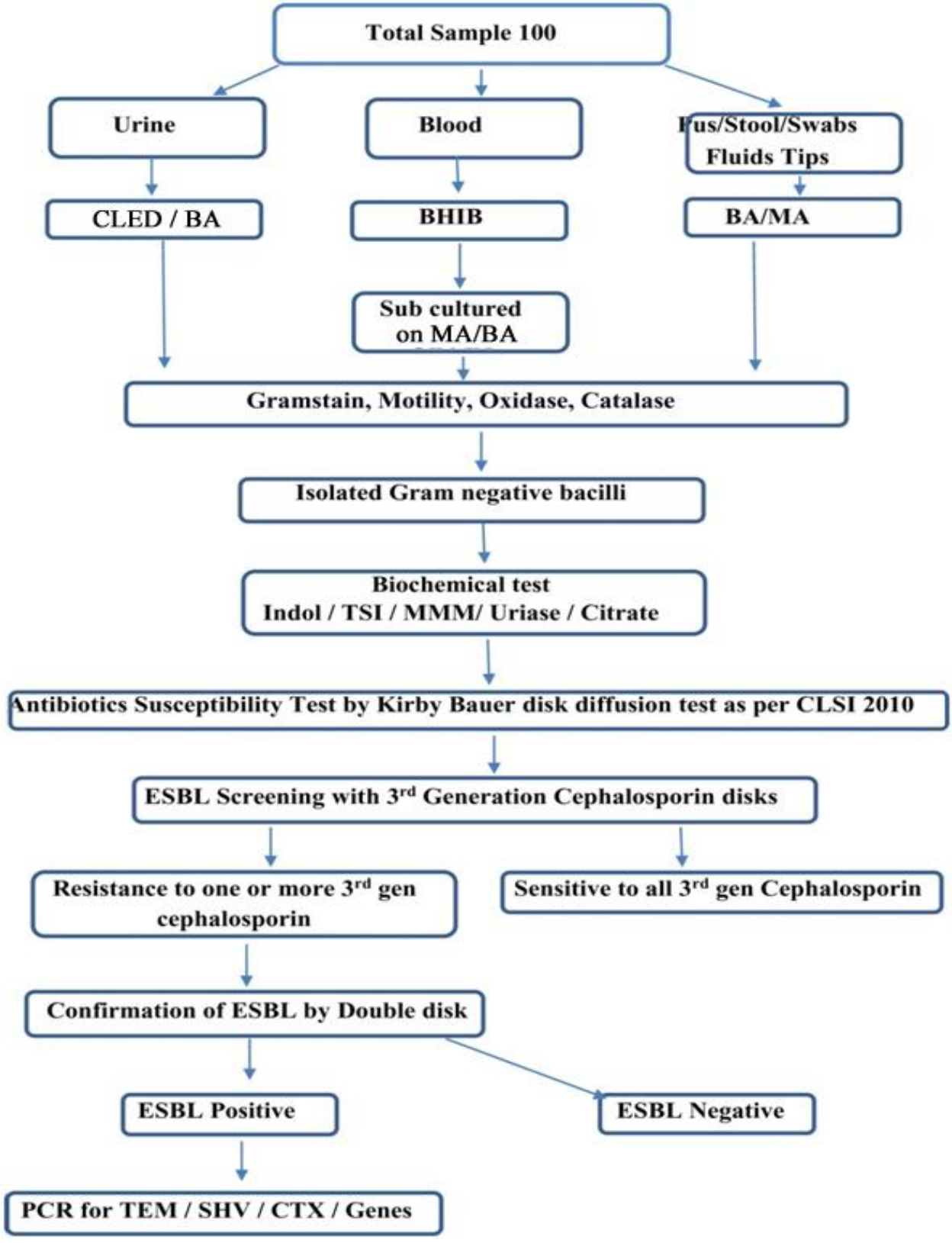


Figure 20: Urea hydrolysis test. **(**PC-Positive control, T-Test, NC-Negative control)

Properties used to characterize *Esch. coli K. pneumoniae and K.oxytoca* are shown below:

Properties	Esch. coli	K. pneumoniae	K.oxytoca
Gram stain	Gram negative bacilli	Gram negative bacilli	Gram negative bacilli
Motility	Motile	Non-motile	Non-motile
Catalase test	Positive	Positive	Positive
Oxidase test	Negative	Negative	Negative
Indole test	Positive	Negative	Positive
TSI	A/A G^+ H_2S^-	A/A G^+ H_2S^-	A/A G^+ H_2S^-
MMannital motility test	M^+M^+	M^+M^-	M^+M^-
Citrate utilization test	Negative	Positive	Positive
Ureas test	Negative	Positive	Positive

Table 3: characters of Esch. coli K. pneumoniae and K.oxytoca



Flowchart 1: Methodology of study

Maintenance of culture strains

Organisms grown in appropriate media for 18 hours were preserved in a nutrient agar slant at 2-8^0 C in a refrigerator and this culture were sub cultured every 2 weeks and maintained till the end of this study. These cultures were used for all the tests.[108,112]

Antimicrobial susceptibility

Kirby-Bauer sensitivity testing method was used for antibiotic sensitivity. From the nutrient agar slant pure cultures of bacteria was grown overnight on MacConkey agar, a suspension matching 0.5 McFarland standard (1.5 x 10^8CFU/ml) was made in nutrient broth. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, the above mentioned disks were placed on the surface and the plates were incubated in air at 37^{o}C for 18-24 hours. The diameters of the zone of inhibition around the disks were measured and recorded Antimicrobial discs used for Gram negative bacteria were Ampicillin 10 µg(AMP), Cephotaxime 30 µg(CTX/CF), Ceftriaxone 30 µg(RP), Ceftazidime 30µg(FG), Gentamicin 10 µg(GM), Amikacin 30 µg(AK), Ciprofloxacin 5 µg(RC), Imipenem 10 µg(IM), Cefoxitin 30µg(CK), Cefepime30 µg(ZX).[107,109,111]

Susceptibility breakpoints of antibiotics tested by disk diffusion method

Antibiotics		Zone diameter		
	Strength	S (mm)	IM (mm)	R (mm)
Cefoxitin (CK)	30µg	≥18	15-17	≤ 14
Ceftazidime (FG)	30µg	≥21	18-20	≤ 17
Cefotaxime (CTX/CF)	30µg	≥26	23-25	≤ 22
Ceftriaxone (RP)	30µg	≥23	20-22	≤ 19
Cefepime (ZX)	30µg	≥18	15-17	≤ 14
Ampicillin (AMP)	10 µg	≥17	14-16	≤ 13
Gentamicin (GM)	5 µg	≥15	13-14	≤ 12
Amikacin (AK)	30µg	≥17	15-16	≤ 14
Ciprofloxacin (RC)	5 µg	≥21	16-20	≤ 15
Imipenum (IM)	10 µg	≥23	20-22	≤ 19

S-sensitive, IM- Intermediate, R-Resistant

Table 4: Antibiotics tested by disk diffusion method

Screening test for ESBLs

Resistance to cefotaxime, ceftazidime, ceftriaxone and aztreonam was detected by disk diffusion test as recommended by CLSI. From the pure cultures of bacteria grown overnight on MacConkey agar, a suspension matching 0.5 McFarland standard (1.5 x 10^8 CFU/ml) was made in nutrient broth. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, the antibiotic disks were placed on the surface and the plates were incubated at 37^{o}C for 18-24 hours. Following growth, the diameter of the zone of inhibition around the disks were measured and recorded.

The disk potency and inhibition zone diameters used for inferring resistance is displayed below. Resistance to at least one of the antibiotics was considered as positive in the screening test for possible ESBL production as per 2010 CLSI guidelines.[44,83,116]

Zone diameters for inferring resistance in the screening test

Antibiotic disk	Resistant, if zone diameter is
cefotaxime (30 µg)	≤ 27 mm
ceftriaxone (30 µg)	≤ 25 mm
ceftazidime (30 µg)	≤ 22 mm

Table 5: Resistance in the screening test

Confirmatory test for ESBLs

Double disk diffusion method (DDDT)

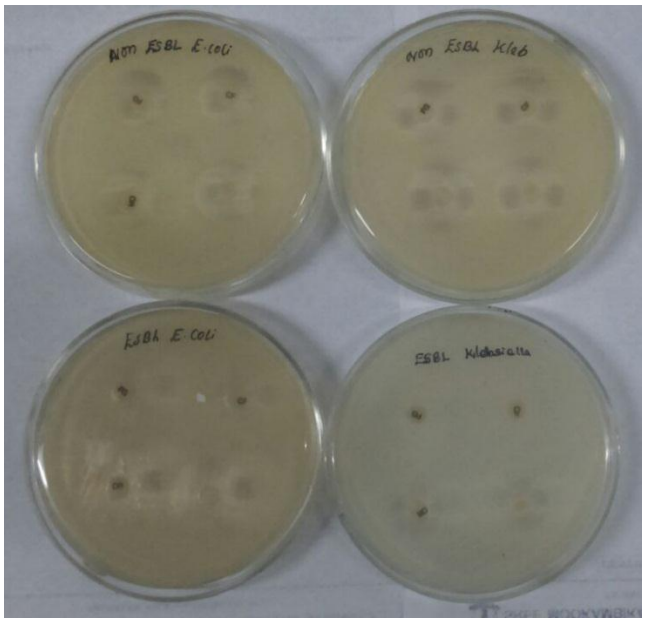


Figure 21: Double disk diffusion method (DDDT)

Isolates of *Esch. coli* and *Klebsiella* that were considered to be positive for ESBL production by the screening test were subjected to the Phenotypic Confirmatory Test (CLSI-PCT) as recommended by 2010 CLSI guidelines.[118]From the pure cultures of bacteria grown overnight on MacConkey agar, a suspension matching 0.5McFarland standard (1.5 x 10^8 CFU/ml) was made in nutrient broth. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, disks of ceftazidime (30 μg) (FG), ceftazidime + clavulanicacid (30/10 μg) (CA), cefotaxime (30 μg) (CTX), cefotaxime + clavulanic acid (30/10μg) (CEC) were placed on the surface and the plates were incubated in air at 37^{o}Cfor 18-24 hours. Following growth, the diameter of the zones around the disks were measured and recorded. An increase in the zone diameter by ≥5 mm around the disks containing cephalosporin with clavulanic over the disks containing cephalosporin alone indicated ESBL production.[112,116,118]

Controls:

Esch. coli ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as controls.

Detection of ESBL genes from bacterial isolates

Material:

PureFast® Bacterial DNA minispin purification kit [Kit contains Lysozyme, Lysozym edigestion buffer, Proteinase-K, Binding buffer, Wash Buffer-1, Wash Buffer-2, Spin columns with collection tube and elution buffer. HELINI 2X ReDdye

PCR Master Mix, Agarose gel electrophoresis consumables and SHV, TEM and CTX Primers are from HELINI Bio-molecules, Chennai, India.

2X Master Mix:

It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and RedDye PCR additives.

Agarose gel electrophoresis:

Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide are from HELINI Bio-molecules, Chennai.

PCR:

- HELINI Ready to use SHV gene Primer mix - 5µl/reaction

PCR Product: 276bp

- HELINI Ready to use TEM gene Primer mix - 5µl/reaction

PCR Product: 250bp

- HELINI Ready to use CTX gene Primer mix - 5µl/reaction

PCR Product: 296bp

Bacterial DNA Purification

1ml of overnight culture
↓ centrifuged at 6000rpm for 5min
Supernatant discarded
↓ Pellet is suspended in 0.2ml PBS
Added 180µl of Lysozyme digestion buffer and 20µl of Lysozyme [10mg/ml]
↓ Incubated at 37C for 15min
Added 400µl of Binding buffer, 5µl of internal control template and 20µl of Proteinase K and Mixed well by inverting several times.
↓ Incubate at 56ºC for 15min
Added 300µl of Ethanol and mixed well.
↓
Transferred entire sample into the PureFast® spin column.
↓ Centrifuged for 1 min
Discard the flow-through and place the column back into the same collection tube.
↓
Added 500µl Wash buffer-1 to the PureFast® spin column.
↓ Centrifuge for 30-60 seconds
Discard the flow-through.
↓ Place the column back into the same collection tube.
Added 500µl Wash buffer-2 to the PureFast® spin column.
↓ Centrifuge for 30-60 seconds
Discard the flow-through
↓
Place the column back into the same collection tube.
↓
Discard the flow-through
↓ centrifuge for an additional 1 min.
(This step is essential to avoid residual ethanol)
↓
Transferred the PureFast® spin column into a fresh 1.5 ml micro-centrifuge tube.
↓
Added 100µl of Elution Buffer to the center of PureFast® spin column membrane.
↓
Incubate for 1 min at room temperature
↓ centrifuge for 2 min
Discard the column and store the purified DNA at -20°C

Flowchart 2: Bacterial DNA Purification

Quality and Quantity of extracted DNA is checked by loading in 1% agarose gel and 5µl of extracted DNA is used for PCR amplification.

PCR Procedure:

- Reactions set up as follows;

Components	Quantity
HELINI RedDye PCR Master mix	10µl
HELINI Ready to use – Primer Mix	5µl
Purified Bacterial DNA	**5µl**
Total volume	20µl

Table 6: PCR Procedure

- Mixed gently and spin down briefly.
- Place into PCR machine and program it as follows;
- Initial De-naturation: 95ºC for 5 min
- De-naturation: 94ºC for 30sec
- Annealing: 58ºC for 30sec
- Extension: 72ºC for 30sec

} 35 cycles

- Final extension: 72º C for 5 min

Loading:

1. Prepared 2% agarose gel. [2gm of agarose in 100ml of 1X TAE buffer]
2. Run electrophoresis at 50V till the dye reaches three fourth distances and observe the bands in UV Transilluminator.

Agarose gel electrophoresis:

1. Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven)
2. When the agarose gel temperature was around 60ºC, added 5µl of Ethidium bromide.
3. Poured warm agarose solution slowly into the gel platform.
4. Kept the gel set undisturbed till the agarose solidifies.
5. Poured 1XTAE buffer into submarine gel tank.
6. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above than the gel.
7. PCR Samples are loaded after mixed with gel loading dye along with 10µl HELINI 100bp DNA Ladder. [100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp and 1500bp]
8. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel.
9. Gel viewed in UV Transilluminator and observed the bands pattern.

Agarose gel electrophoresis bands pattern

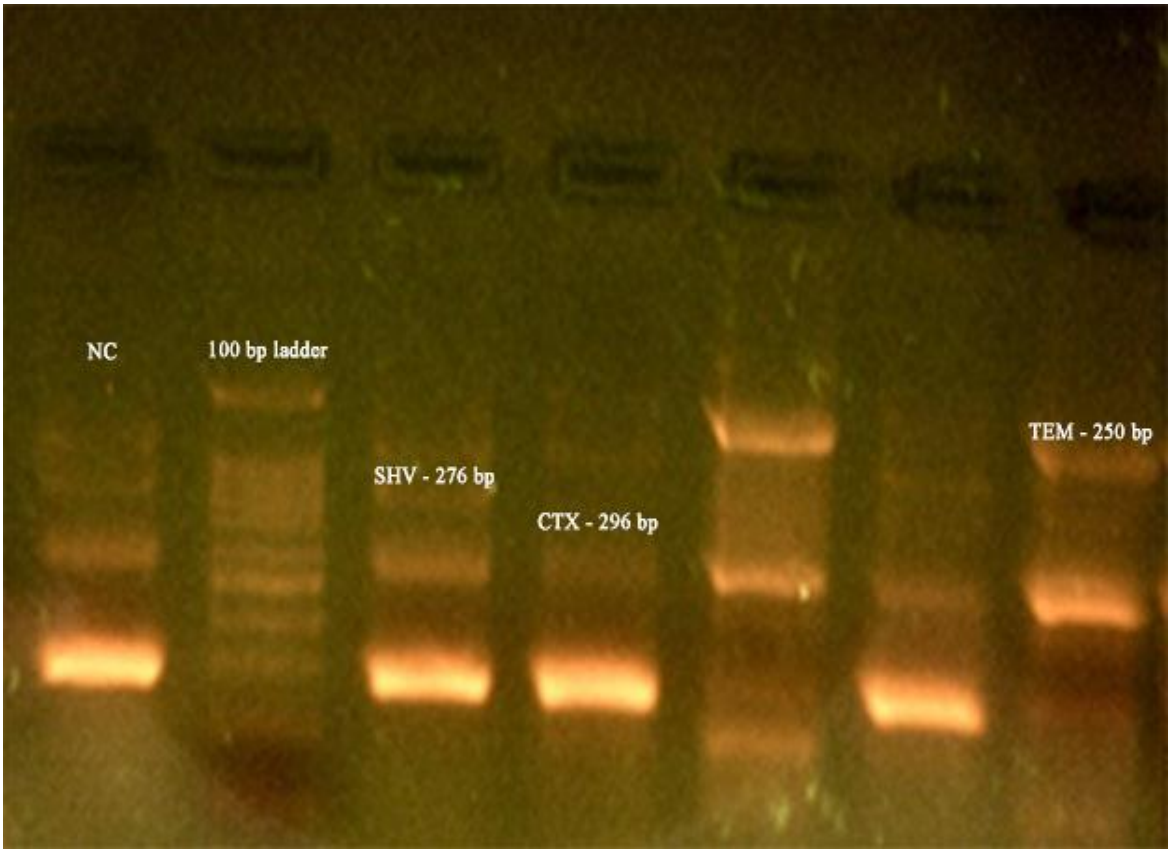


Figure 22: Agarose gel electrophoresis bands pattern

Results

RESULTS

Clinical samples

- During the one year period from the total number of gram negative bacilli isolates first 50 isolates of Esch.*coli* and 50 isolates of *Klebsiella* were cultured from various clinical samples.
- All the samples were collected under strict aseptic precaution to avoid cross contaminations.
- All the samples were cultured soon after reaching the laboratory.
- The urine samples were inoculated in CLED and BA, blood was inoculated in to BHIB and pus, sputum, swabs fluids and tips were inoculated in to BA and MA.
- Blood samples were sub cultured after overnight inoculation and on 5^{th} day in to the MA and BA for isolation. In between bottles are examined daily and sub-cultured into solid media if there is any visible sign of growth. The plates were incubated at 37^{0}c for 24 hours.
- After 24 hours period the plates were observed, the colony and organism was confirmed by gram stain and biochemical tests.
- The distribution of the various samples and its percentage from them is displayed in the Table. Urine 65 (65%), Sputum 11 (11%), Stool 9(9%) and pus 8 (8%) were the common samples submitted for culture.

Table 7: Distribution of sample from various specimens

Clinical sample	Total number of samples n=100	Percentage (%)
Urine	65	65%
Sputum	11	11%
Stool	9	9%
Pus	8	8%
Blood	1	1%
Suction tip	1	1%
ET tip	1	1%
Vaginal swab	1	1%
Ear swab	1	1%
Ascitic fluid	1	1%
Pleural fluid	1	1%

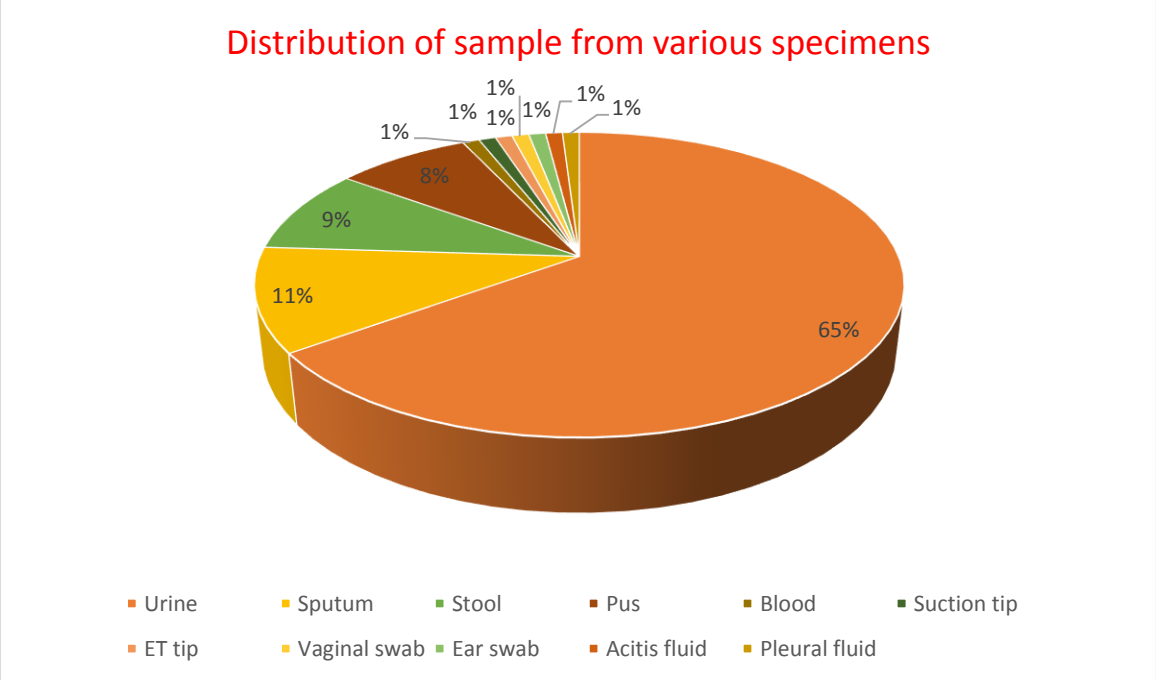


Figure 23: Distribution of sample from various specimens

Among 100 clinical isolates 65 (65%) samples were from Urine followed by 11 (11%) from sputum, 9 (9%) from Stool, 8 (8%) from pus. Others from blood, suction tip, ET tip, vaginal swab, ascitic fluid, ear swab and pleural fluid were 1 (1%) each.

Table 8: Detection rate of Esch.coli from the various samples

Clinical sample	Esch.coli n=(50)	Percentage (%)
Urine	39	**78%**
Sputum	**2**	**4%**
Pus	**3**	**6%**
Stool	**5**	**10%**
Blood	**0**	**0%**
Suction tip	**0**	**0%**
ET tip	**0**	**0%**
Vaginal swab	**0**	**0%**
Ear swab	**0**	**0%**
Ascitic fluid	**1**	**2%**
Pleural fluid	**0**	**0%**

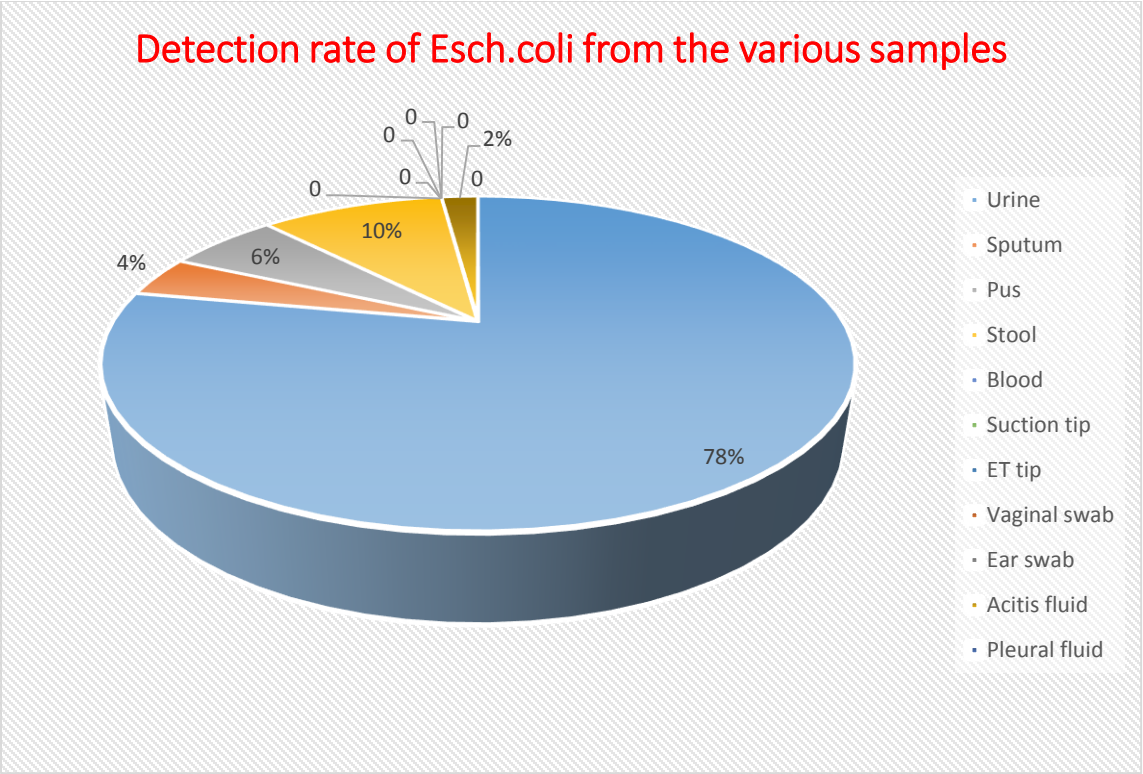


Figure 24: Detection rate of Esch.coli from the various samples

Among 50 isolates of *Esch.coli* 39(78%) samples were obtained from urine followed by 5 (10%) from stool, 3 (6%) from pus, 2 (4%) from sputum and 1 (2%) from ascitic fluid.

Table 9: Detection rate of Klebsiella from the various samples

Clinical sample	Klebsiella n=(50)	Percentage (%)
Urine	26	52%
Sputum	9	18%
Pus	5	10%
Stool	4	8%
Blood	1	2%
Suction tip	1	2%
ET tip	1	2%
Vaginal swab	1	2%
Ear swab	1	2%
Ascitic fluid	0	0%
Pleural fluid	1	2%

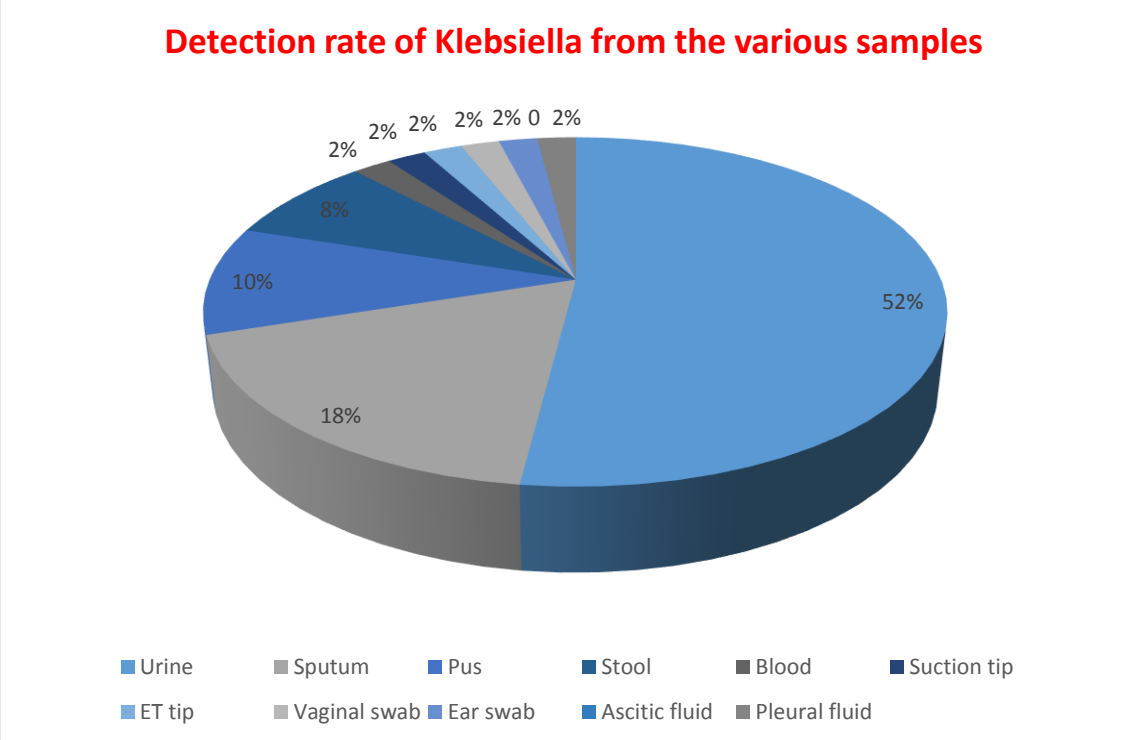


Figure 25 : Detection rate of Klebsiella from the various samples

Among 50 isolates of Klebsiella isolates 26 (52%) were obtained from Urine, 9(18%) from sputum, 5 (10%) pus, 4(8%) stool. 1 (2%) from others like blood, suction tip, ET tip, vaginal swab, ear swab and pleural fluid.

Anti-Biogram

Table 10: Antibiotic susceptibility of *Esch.coli*

Antibiotics	Esch.coli(50)		
	Sensitive	Intermediate	Resistant
Cefoxitin 30µg (CK)	15 (30%)	-	35 (70%)
Ceftazidim30µg (FG)	15 (30%)	-	35 (70%)
Cefotaxim 30 µg (CTX/CF)	15 (30%)	-	35 (70%)
Ceftriaxone30µg (RP)	15 (30%)	-	35 (70%)
Cefepime30 µg(ZX)	21 (42%)	3 (6%)	26 (52%)
Ampicillin10 µg(AMP)	22 (44%)	-	28 (56%)
Gentamicin5 µg(GM)	28 (56%)	4 (8%)	18 (36%)
Amikacin30 µg(AK)	44 (88%)		6 (12%)
Ciprofloxacin5 µg(RC)	15 (30%)		35 (70%)
Imipenum10 µg(IM)	36 (72%)	6 (12%)	8 (16%)

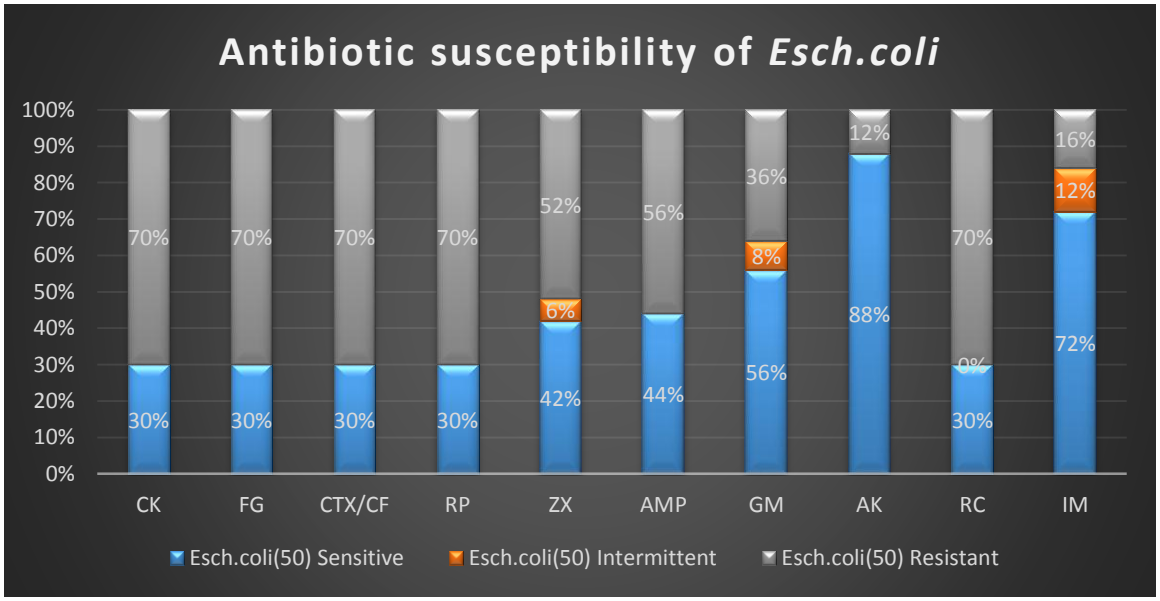


Figure 26: Antibiotic susceptibility of Esch.coli

Above table and figure shows antibiotic sensitivity and resistant pattern among the 50 Esch.coli isolates.

- Among 50 isolates of Esch.coli 30 (70%) show resistance to cefoxitin (CK), 30 (70%) to ceftazidime (FG), 30 (70%) to ceftriaxone (RP), 30 (70%) to cefotaxime (CTX/CF), 30 (70%) to Ciprofloxacin (RC), 6 (12%) to Amikacin(AK), 28 (56%) to Ampicillin(AMP), 26 (52%) to Cefepime (ZX), 18 (36%) to Gentamicin (GM) and 8 (16%) to Imipenem (IM).
- In this study 30 (70%) of Esch.coli show high resistance to cefoxitin (CK), ceftazidime (FG), ceftriaxone (RP), cefotaxime (CTX/CF) and Ciprofloxacin (RC).
- Within this 50 Esch.coli 44 (88%) show high sensitivity to Amikacin (AK) and 36 (72%) to Imipenum (IM).
- Among this 40-50% show sensitivity to Cefepime (ZX), Gentamicin (GM), Ampicillin (AMP).
- 3 (6%) of Esch.coli show intermediate sensitivity to Cefepime (ZX), 4 (8%) to Gentamicin (GM) and 6 (12%) to Imipenum (IM).

Table 11: Antibiotic susceptibility of *Klebsiella*

Antibiotics	Klebsiella (50)		
	Sensitive	Intermediate	Resistant
Cefoxitin 30µg (CK)	27 (54%)	-	23 (46%)
Ceftazidim30µg (FG)	27 (54%)	-	23 (46%)
Cefotaxim 30 µg (CTX/CF)	27 (54%)	-	23 (46%)
Ceftriaxone30µg (RP)	27 (54%)	-	23 (46%)
Cefepime30 µg(ZX)	37 (74%)	3 (6%)	10 (20%)
Ampicillin10 µg(AMP)	35 (70%)	-	15 (30%)
Gentamicin5 µg(GM)	17 (34%)	-	33 (66%)
Amikacin30 µg(AK)	40 (80%)	-	10 (20%)
Ciprofloxacin5 µg(RC)	27 (54%)	-	23 (46%)
Imipenum10 µg(IM)	41 (82%)	2 (4%)	7 (14%)

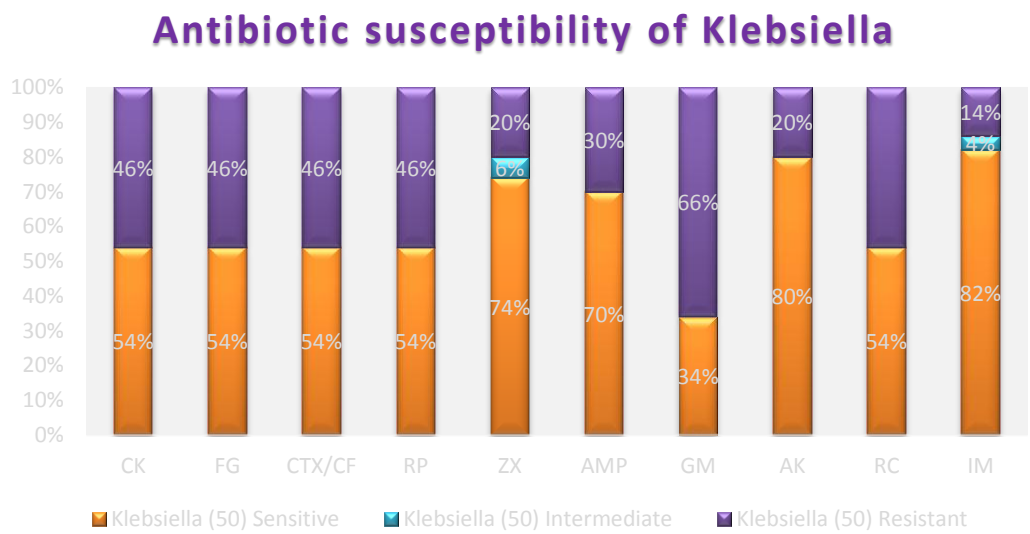


Figure 27: Antibiotic susceptibility of Klebsiella

- Among 50 isolates of Klebsiella 23 (46%) were show resistance to cefoxitin (CK), 23 (46%) to ceftazidime (FG), 23 (46%) to ceftriaxone (RP), 23

(46%) to cefotaxime (CTX/CF), 23 (46%) Ciprofloxacin (RC), 33(66%) Gentamicin (GM), 7 (14%) to Imipenum (IM), 10 (20%) Cefepime (ZX), 10 (20%) to Amikacin (AK) and 15 (30%) to Ampicillin (AMP).

- In this study 33(66%) Gentamicin (GM) shows high resistance followed by 23 (46%) to cefoxitin (CK), ceftazidime (FG), ceftriaxone (RP), cefotaxime (CTX/CF), Ciprofloxacin (RC).
- In this study 41 (82%) of klesiella show high sensitivity to Imipenum (IM) and 40 (80%) to Amikacin (AK).
- Among this 70-75% show sensitivity to Cefepime (ZX) and Ampicillin (AMP).
- 3 (6%) Klesiella show intermediate sensitivity to Cefepime (ZX) and 2 (4%) to Imipenum (IM).

Table 12: Screening test for ESBL production with 3rd Generation cephalosporins

	Ceftazidim30µg (FG)		Cefotaxim 30 µg (CTX/CF)		Ceftriaxone30µg (RP)	
	S	**R**	**S**	**R**	**S**	**R**
Esch.coli (50)	**15 (30%)**	**35 (70%)**	**15 (30%)**	**35 (70%)**	**15 (30%)**	**35 (70%)**
Klebsiella (50)	**27 (54%)**	**23 (46%)**	**27 (54%)**	**23 (46%)**	**27 (54%)**	**23 (46%)**
Total (100)	**42 (42%)**	**58 (58%)**	**42 (42%)**	**58 (58%)**	**42 (42%)**	**58 (58%)**

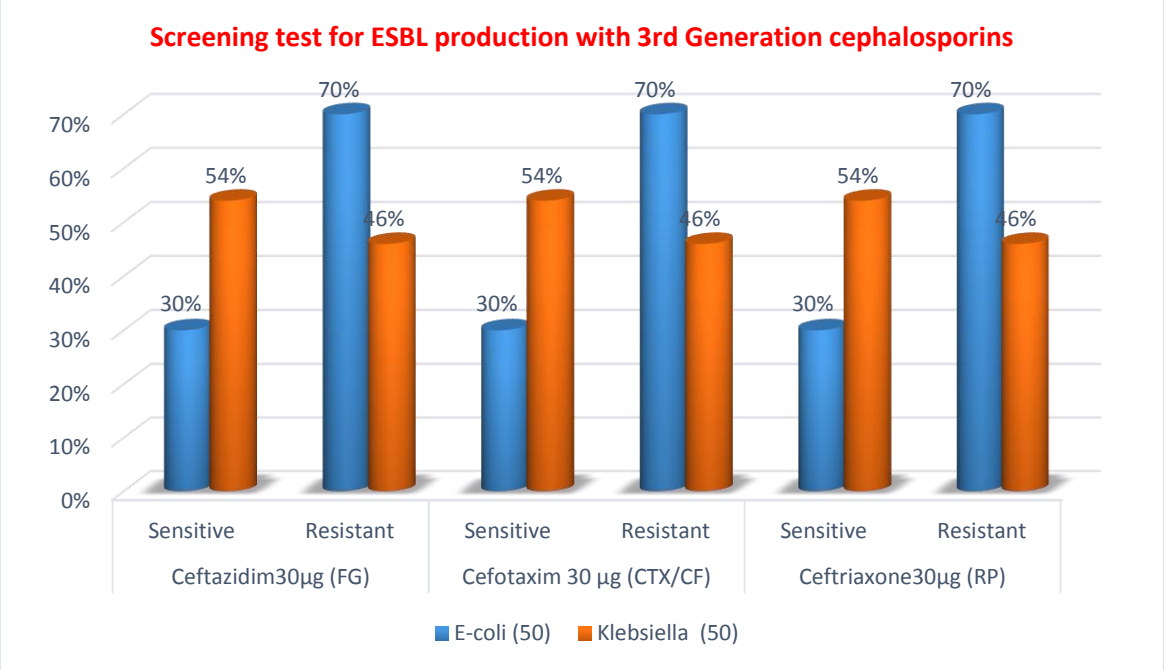


Figure 28: Screening test for ESBL production with 3rd Generation cephalosporins

Screening test for ESBL production

- Screening test involved detection of resistance to one or more of the 3rdGeneration cephalosporin antibiotics (ceftazidime, cefotaxime, ceftriaxone).
- Among 100 isolates 58(58%) isolates were found to be resistant to all the three 3rd Generation cephalosporins.
- Among the 50 Esch.coli isolates, 35(70%) were found to be resistant to all the three screening agents.
- In the total 50Klebsiella isolates, 23 (46%) were found to be resistant to all the three screening agents. Among these 2(8.7%) were K.oxytoca and were as 21 (91.3%) were K.pneumonia.

Table 13: Confirmation of ESBL production by combination Disk diffusion test:

	CAZ/CAZC		CTX/CTXC	
Name of organism	Sensitive	Resistant	Sensitive	Resistant
Esch. coli *n= 50*	15 (30%)	35 (70%)	15 (30%)	35 (70%)
Klebsiella spp*n= 50*	27 (54%)	23 (46%)	27 (54%)	23 (46%)
Total n = 100	42 (42%)	58 (58%)	42 (42%)	58 (58%)

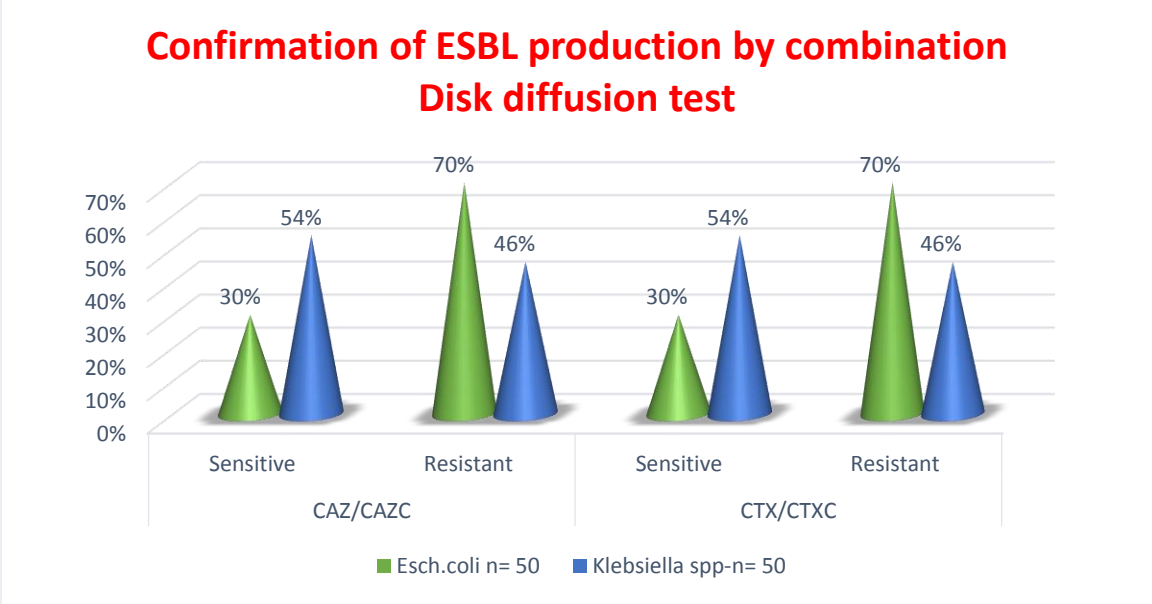


Figure 29: Confirmation of ESBL production by combination Disk diffusion test:

<u>Confirmation of ESBL production by combination Disk diffusion test:</u>

- All the 58 isolates that were positive in the ESL screening test, were positive in the confirmative test by combination disk diffusion test also.
- Among 50 Esch.coli 35(70%) isolates were positive in the screening test, and all of them 35 (70%) were phenotypically confirmed as ESBL producers.

- Similarly, from the 50Klebsiella isolates, 23(46%) were identified as ESBL producers and all the 23 (46%) isolates were confirmed as ESBL phenotypically. Among 23 Klebsiella isolates 2 (8.7%) were K.oxytoca and 21 (91.3%) were K.pneumoniae.

Table 14: Antibiotic resistance pattern among ESBL producers

	Esch.coli			Klebsiella		
	S	IM	R	S	IM	R
CK	0 (0%)	0 (0%)	35 (100%)	0 (0%)	0 (0%)	23 (100%)
ZX	6 (17.14%)	3 (8.57%)	26 (74.2%)	10 (43.47%)	3 (13.04%)	10 (43.47%)
AMP	15 (42.85%)	0 (0%)	20 (57.14%)	15 (65.21%)	0 (0%)	8 (34.78%)
GM	15 (42.85%)	3 (8.57%)	17 (48.57%)	11 (47.82%)	0 (0%)	12 (52.17%)
AK	30 (85.71%)	0 (0%)	5 (14.28%)	18 (78.26%)	0 (0%)	5 (21.73%)
RC	0 (0%)	0 (0%)	35 (100%)	0 (0%)	0 (0%)	23 (100%)
IMP	21 (60.00%)	6 (17.14%)	8 (22.85%)	14 (60.86%)	2 (8.69%)	7 (30.43%)

Above table shows antibiotic sensitivity and resistant pattern among the ESBL producing Esch.coli (35) and Klebsiella isolates (23) to identify the co-resistance antibiotics.

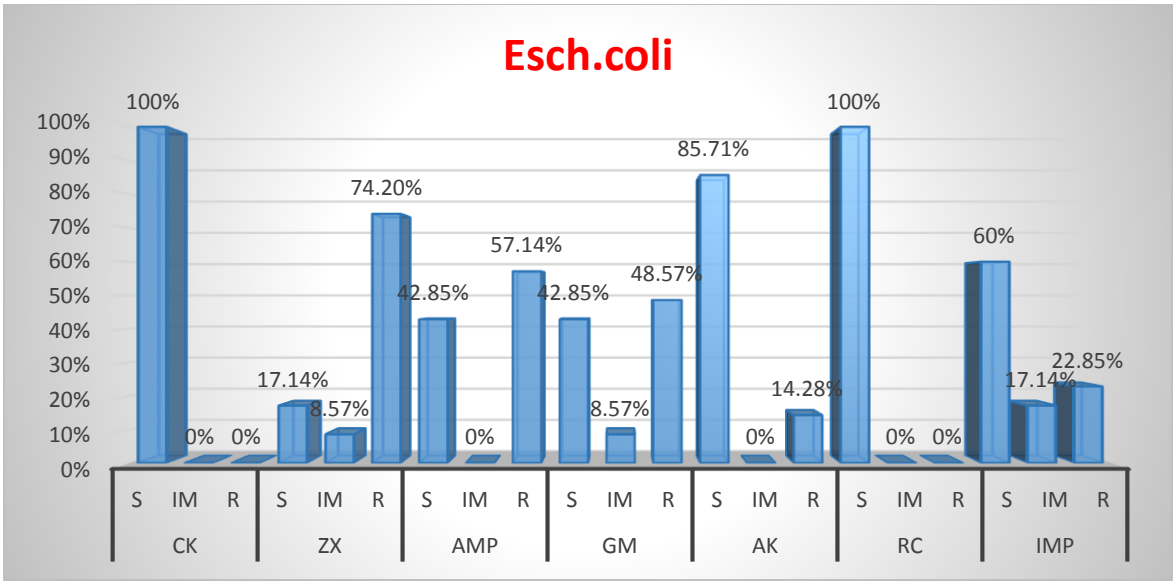


Figure 30: Antibiotic resistance pattern among ESBL producers – Esch.coli

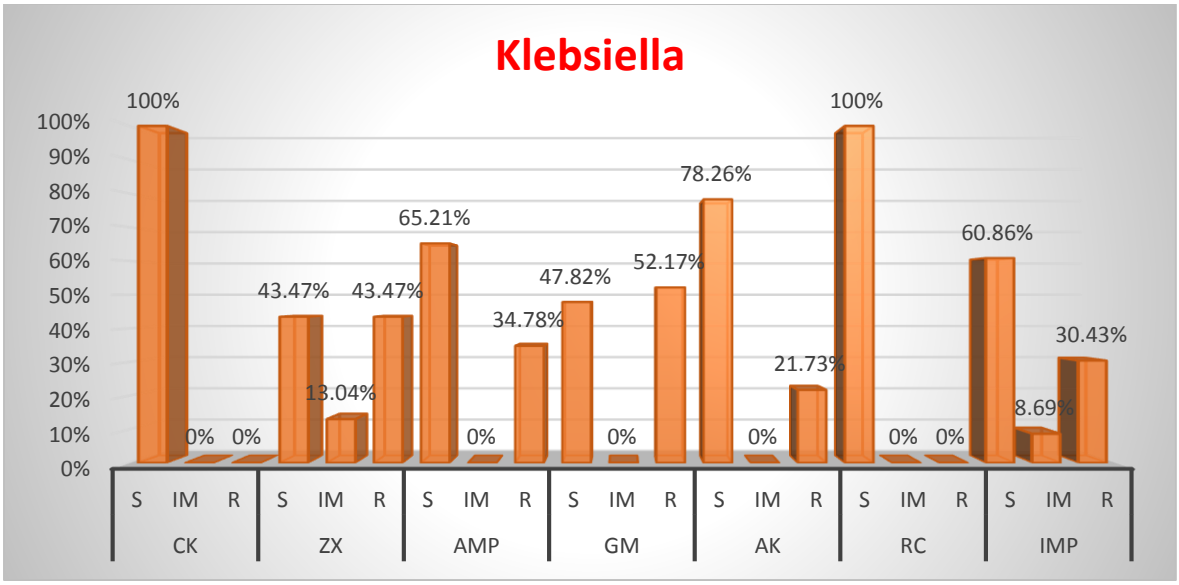


Figure 31: Antibiotic resistance pattern among ESBL producers – Klebsiella

Antibiotic resistance pattern among ESBL producers:

- Among 35 ESBL Esch.coli isolates all the 35 (100%) were resistance to cefoxitin (CK), 35 (100%) to Ciprofloxacin (RC), 5 (14.28%) to Amikacin (AK), 20 (57.14%) to Ampicillin (AMP), 26 (74.2%) to Cefepime (ZX), 17 (48.57%) to Gentamicin (GM) and 8 (22.85%) to Imipenum (IM).
- In this study ESBL all 35 (100%) Esch.coli isolates show resistance to cefoxitin (CK) and Ciprofloxacin (RC).
- Among the 35 ESBL isolates 85.71% show high sensitivity to Amikacin (AK) and 60% to Imipenum (IM).
- In this study ESBL isolates show 45% - 60% sensitivity to Ampicillin (AMP) and Gentamicin (GM).
- Among ESBL isolates3 (8.57%) show intermediate sensitivity to Cefepime (ZX), 3 (8.57%) Gentamicin (GM) and 6 (17.14%) to Imipenum (IM).
- Among 23 ESBL Klebsiella isolates 23 (100%) show resistance to cefoxitin (CK), 23(100%) to Ciprofloxacin (RC), 5 (21.73%) to Amikacin (AK), 8 (34.78%) to Ampicillin (AMP), 10 (43.47%) to Cefepime (ZX), 12 (52.17%) Gentamicin (GM) and 7 (30.43%) Imipenum (IM).
- In this study all the 23 (100%) show resistance to cefoxitin (CK) and Ciprofloxacin (RC) followed by 52.17% Gentamicin (GM).
- Among this 78.2% show high sensitivity to Amikacin(AK), 65.21% Ampicillin(AMP) and 60.86% to Imipenum (IM).

- In this 40% - 50% of ESBL Klebsiella show sensitivity to Cefepime (ZX) and Gentamicin (GM).
- 3 (13.04%) ESBL isolate show intermediate sensitivity to Cefepime (ZX) and 2 (8.69%) Imipenum (IM).

Table 15: Pattern of distribution of TEM, SHV and CTX-M genes among phenotypic confirmed ESBL producers

	TEM	SHV	CTX-M
Esch.coli (35)	**26 (74.28%)**	**21 (60.00%)**	**29 (82.85%)**
Klebsiella (23)	**15 (65.21%)**	**12 (52.17%)**	**19 (82.60%)**
Total (58)	**39 (67.24%)**	**32 (55.17%)**	**47 (81.03%)**

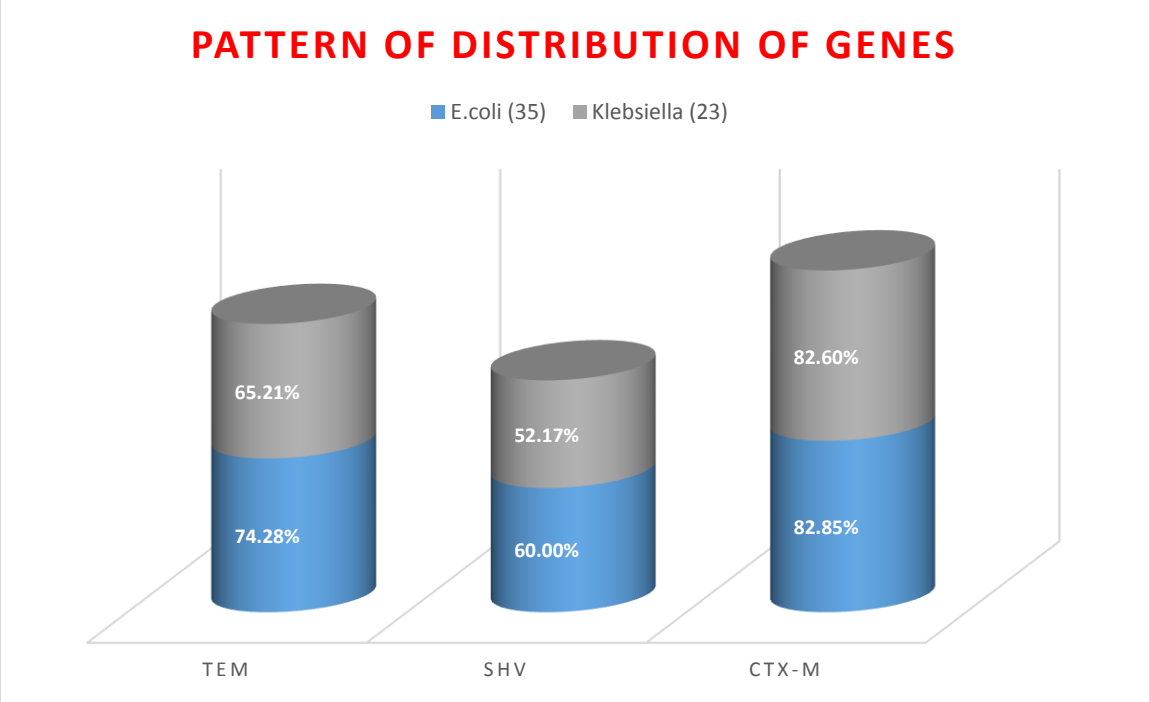


Figure 32: Distribution of Genes

	***bla*-TEM+*bla*-SHV**	***bla*-SHV+*bla*-CTX**	***bla*-TEM+*bla*-CTX**	***bla*-TEM+*bla*-SHV+*bla*-CTX**
Ech.coli	12 (34.28%)	15(42.85%)	20(57.1%)	6(17.1%)
Klebsiella	4(17.39%)	8(34.78%)	11(47.82%)	0 (0%)

Table 16: Pattern of distribution of bla-TEM, bla-SHV and bla-CTX-M genes among phenotypic confirmed ESBL producers

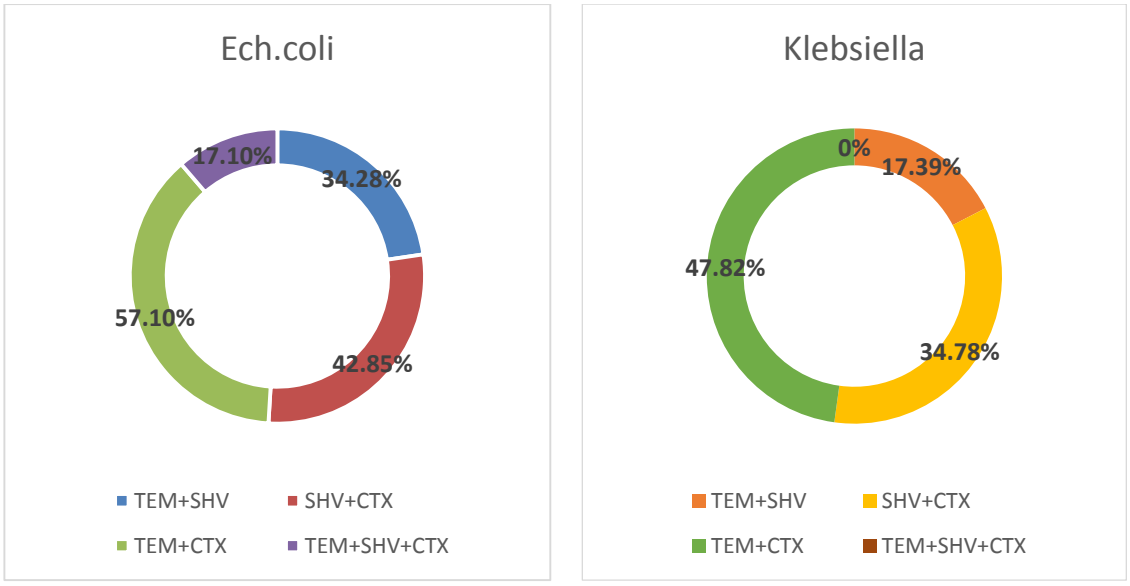


Figure 33: Pattern of distribution of Genes with Esch.coli and Klebsiella

Pattern of distribution of *bla*-TEM, *bla*-SHV and *bla*-CTX-M genes among phenotypic confirmed ESBL producers

- An attempt to detect one or more bla genes by PCR in (58) all the phenotypically confirmed ESBL producers.
- This included 35(60.3%) isolates of Esch. coli and 23 (39.7%) isolates of Klebsiella.

- Among 58 ESBL producers *bla*-TEM gene was detected in 39 (67.2%), *bla*-SHV gene in 32 (55.17%) and *bla*-CTX-M gene in 47(81.03%) were detected.
- Among 35 ESBL Esch.coli isolates, 26 (74.28%) were positive for *bla*-TEM gene, 21 (60%) were *bla*-SHV gene and 29(82.85%) were *bla*-CTX-M gene.
- All the isolates were shows combined genes *bla*-TEM+*bla*-SHV 12(34.3%), *bla*-SHV+*bla*-CTX-M 15(42.8%), *bla*-TEM+*bla*-CTX-M 20(57.1%) and *bal*-TEM+*bla*-SHV+*bla*-CTX-M 6(17.1%).
- In all the ESBL producing Esch.coli isolates more than one gene was detected.
- Among 23 ESBL Klebsiella isolates included 15(65.21%) were *bla*-TEM, 12(52.17%) were *bla*-SHV and 19 (82.60%) were *bla*-CTX-M. All the isolates were shows complaint genes *bla*-TEM+*bla*-SHV 4(17.39%), *bla*-SHV+*bla*-CTX-M 8 (34.78%), and *bla*-TEM+*bla*-CTX-M 11 (47.82%).
- In all the ESBL producing Klesiella isolatates more than one gene was detected.

Table 17: Relationship between Antibiotic resistance patterns with bla genes of Esch.coli

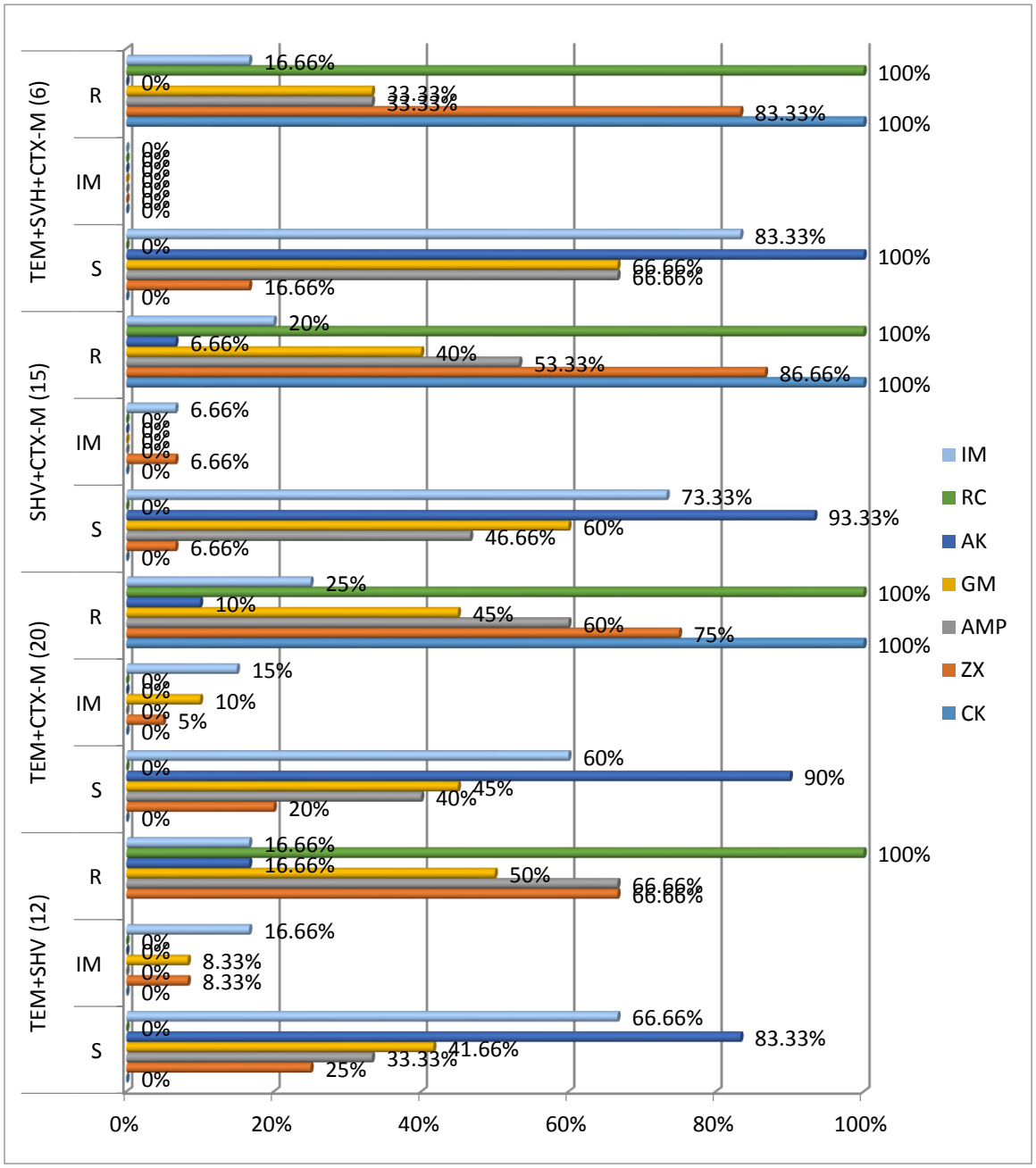
Antibiotics	TEM+SHV (12)			TEM+CTX-M (20)			SHV+CTX-M (15)			TEM+SVH +CTX-M (6)		
	S	IM	R	S	IM	R	S	IM	R	S	IM	R
CK	0 0%	0 0%	12 100%	0 0%	0 0%	20 100 %	0 0%	0 0%	15 100%	0 0%	0 0 %	6 100%
ZX	3 25%	1 8.33%	8 66.66 %	4 20 %	1 5%	15 75%	1 6.66%	1 6.66 %	13 86.66 %	1 16.66 %	0 0 %	5 83.33 %
AMP	4 33.33 %	0 0%	8 66.66 %	8 40 %	0 0%	12 60%	7 46.66 %	0 0%	8 53.33 %	4 66.66 %	0 0 %	2 33.33 %
GM	5 41.66 %	1 8.33%	6 50%	9 45 %	2 10 %	9 45%	9 60%	0 0%	6 40%	4 66.66 %	0 0 %	2 33.33 %
AK	10 83.33 %	0 0%	2 16.66 %	18 90 %	0 0%	2 10%	14 93.33 %	0 0%	1 6.66%	6 100%	0 0 %	0 0%
RC	0 0%	0 0%	12 100%	0 0%	0 0%	20 100 %	0 0%	0 0%	15 100%	0 0%	0 0 %	6 100%
IM	8 66.66 %	2 16.66 %	2 16.66 %	12 60 %	3 15 %	5 25%	11 73.33 %	1 6.66 %	3 20%	58.33 %	0 0 %	1 16.66 %

CK- Cefoxitin **, ZX**- Cefepime**, AMP**-Ampicillin**, GM**- Gentamicin**, AK**-Amikacin**, RC**- Ciprofloxacin**, IM**- Imipenem **. (S**- Sensitive, IM- Intermediate, R-Resistant.)

- Above table shows the relationship between Antibiotic resistance patterns with *bla* genes (*bla* TEM, *bla* SHV, *bla* CTX-M) of ESBL producing Esch.coli in our study.
- In this study all combination of genes show 100% resistance to cefoxitin (CK) and ciprofloxacin (RC).
- Isolates show 80% - 100% sensitivity Amikacin (AK).
- Among this 60% - 70% show sensitive to Imipenum (IM).

- ❖ Among ESBL producing gens 70% - 80% were shows high resistance to Cefipime (ZX).

Figure 34 : Relationship between Antibiotic resistance patterns with bla genes of Esch.coli



CK- Cefoxitin , ZX- Cefepime, AMP-Ampicillin, GM- Gentamicin, AK- Amikacin, RC- Ciprofloxacin, IM- Imipenum.

Table 18 : Relationship between Antibiotic resistance patterns with *bla* genes of Klebsiella:

Antibiotics	TEM+SHV (4)			TEM+CTX-M (8)			SHV+CTX-M (11)			TEM+SVH+ CTX-M (0)		
	S	IM	R	S	IM	R	S	IM	R	S	IM	R
CK	0 0%	0 0%	4 100 %	0 0%	0 0%	8 100 %	0 0%	0 0%	11 100%	0 0%	0 0%	0 0%
ZX	1 25%	1 25%	2 50%	3 37.5 %	0 0%	5 62.5 %	6 54.5 4%	2 18.1 8%	3 27.27%	0 0%	0 0%	0 0%
AMP	2 50%	0 0%	2 50%	4 50%	0 0%	4 50%	10 90.9 0%	0 0%	1 9.09%	0 0%	0 0%	0 0%
GM	2 50%	0 0%	2 50%	5 62.5 %	0 0%	3 37.5 %	4 36.3 6%	0 0%	7 63.63%	0 0%	0 0%	0 0%
AK	3 75%	0 0%	1 25%	5 62.5 %	0 0%	3 37.5 %	10 90.9 0%	0 0%	1 9.09%	0 0%	0 0%	0 0%
RC	0 0%	0 0%	4 100 %	0 0%	0 0%	8 100 %	0 0%	0 0%	11 100%	0 0%	0 0%	0 0%
IM	3 75%	1 25%	0 0%	4 50%	0 0%	4 50%	**7** 63.6 3%	1 9.09 %	3 27.27%	0 0%	0 0%	0 0%

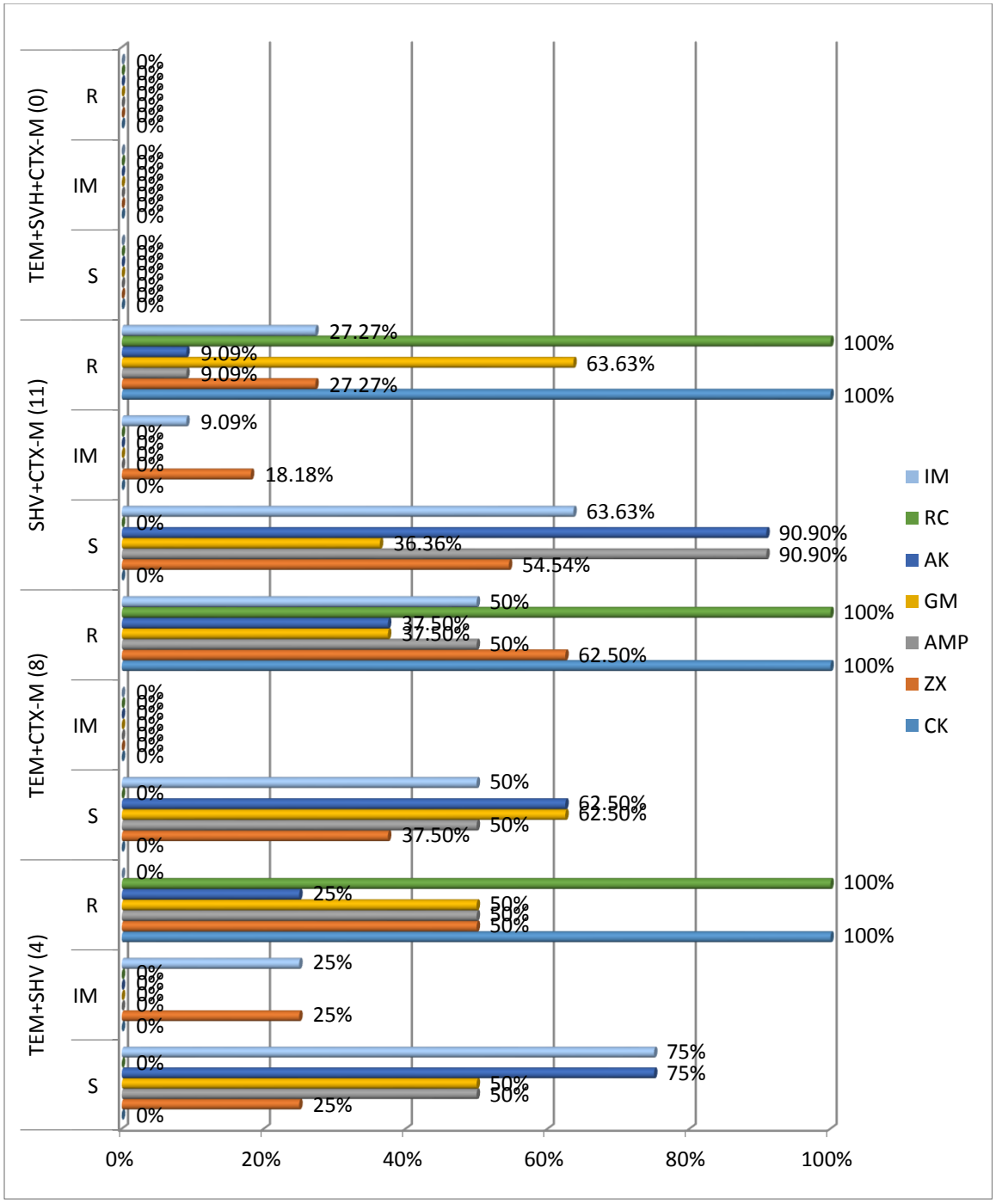
CK- Cefoxitin **, ZX**- Cefepime**, AMP**-Ampicillin**, GM**- Gentamicin**, AK**- Amikacin**, RC**- Ciprofloxacin**, IM**- Imipenum.

S-Sensitive, IM- Intermediate, R- Resistant.

- Above table shows the relationship between Antibiotic resistance patterns with *bla* (*bla* TEM, *bla* SHV, *bla* CTX-M) genes of ESBL producing Klebsiella in our study.
- In this study all combination of genes shows 100% resistance to cefoxitin (CK) and ciprofloxacin (RC).
- Among this 60% - 90% show sensitivity to Amikacin (AK).
- In this 60% - 80% genes show sensitive to Imipenum (IM).

❖ Among the ESBL producing gens 50% - 60% were shows high resistance to Cefipime (ZX).

Figure 35 : Relationship between Antibiotic resistance with bla genes of Klebsiella



CK- Cefoxitin , ZX- Cefepime, AMP-Ampicillin, GM- Gentamicin, AK- Amikacin, RC- Ciprofloxacin, IM- Imipenum.

Discussion

Discussion

- Nowadays antibiotics 3rd Gen. Cephalosporin antibiotics are continuously being added for the treatment of various infections.
- An extensive use of β-lactam antibiotics in hospital and community has created a major problem leading to increased morbidity, mortality and health care costs.
- Proper use of antibiotics is very important to prevent multi drug resistance.
- Prevalence of bacterial resistance against 3rd Gen. Cephalosporin antibiotics makes the main focus of this study.
- In the present study, a total of 100 isolates (50 Esch.coli and 50 Klebsiella) were isolated from various clinical specimens.
- Majority of the organisms were isolated from urine 65% (65/100) followed by Sputum 11% (11/100), Stool 9% (9/100) (Table:7)
- Among 100 clinical isolates 65 (65%) samples were from Urine followed by 11 (11%) from sputum, 9 (9%) from Stool, 8 (8%) from pus. Others from blood, suction tip, ET tip, vaginal swab, ascitic fluid, ear swab and pleural fluid were 1 (1%) each.
- Among 50 isolates of Klebsiella isolates 26 (52%) were obtained from Urine, 9(18%) from sputum, 5 (10%) pus, 4(8%) stool. 1 (2%) from others like blood, suction tip, ET tip, vaginal swab, ear swab and pleural fluid
- The percentage of isolations from various clinical samples correlates with finding done Shiju MP et al (201o), Vipul M Khakhkhar et al (2012),

Archana Sharma et al (2012), Sridhar PN Rao et al (2014) and Raymond G Batchoun et al (2009).[108,110,113,114,115]

- Among Klebsiella isolates the above studies also mention Sputum is the second common isolate which is correlate with present study.
- The organism encoding multiple antibiotic resistance genes are becoming increasingly prevalent. In this study Esch.coli presented with (70%) high resistance to Cefoxitin, 3rd Gen.Cephalosporins (70%) to Ceftazidime, (70%) to Cefotaxim and (70%) to ceftriaxone and (70%) to Ciprofloxacin followed by (56%) to Ampicillin, (50%) Cefepim and (46%) Gentamicin.
- Among this (88%) isolates show high sensitivity to Amikacin and (83%) Imipenum.
- Klebsiella show (66%) high resistance to Gentamicin followed by 2nd Gen. Cephalosorin (46%) to cefoxitin, 3rd Gen. Cephalosporins(46%) to Ceftazidime, (46%) to Cefotaxim, (46%) to ceftriaxone), 46% to Ciprofloxacin and (30%) to Ampicillin.
- Among this (80%) show high sensitivity to Amikacin and (86%) to Imipenem.
- This present study Esch.coli shows high resistance to Cephalosporins (70%) and Ciprofloxacin (70%) whereas Klebsiella shows high resistance to Gentamicin (66%).This result correlate with Archana sharma et al 2012 and Shiju MP et al 2010. [114,115]

- Vipul M Khakhkhar et al 2012 concluded that almost all ESBL producers resistant to ampicillin, but in the present study 42.85% ESBL producing Esch.coli show sensitive to Ampicillin and 65.21% ESBL producing Klebsiella.[113]
- In the present study ESBL producing bacteria shows high sensitivity to Amikacin and Imipenem, which correlates with Shiju MP et al 2010, Vipul M Khakhkhar et al 2012, Archana Sharma et al 2012, Abolfazl Gholipour et al 2014 and ModiDhara et al 2012.[109,111,113,114,115]
- In the present study 74.28% ESBL producing Esch.coli and 43.47% ESBL producing Klebsiella shows resistance to cefepime. Sridar PN Rao et al 2014 which correlate with present study.[110]
- Sridar PN Rao et al 2014 documented significantly more numbers of ESBL-Klebsiella were resistant to cefepime than ESBL-Esch.coli, but the present study ESBL Esch.coli shows 74.28% and ESBL Klebsiella shows 43.47%.[110]
- In the present study, all ESBL producers were uniformly resistant to ceftriaxone, ceftazidime and cefotaxime Esch.coli shows 70% and Klebsiella shows 46%. This data correlate with Sridar PN Rao et al 2014.[110]
- Pitout JDD et al 2008 documented Co-resistance to amino glycosides is common in ESBL-producers, which does not correlates with present study. Present study shows 50 – 65% sensitivity to gentamicin and 75 – 90% to amikacin.[2]

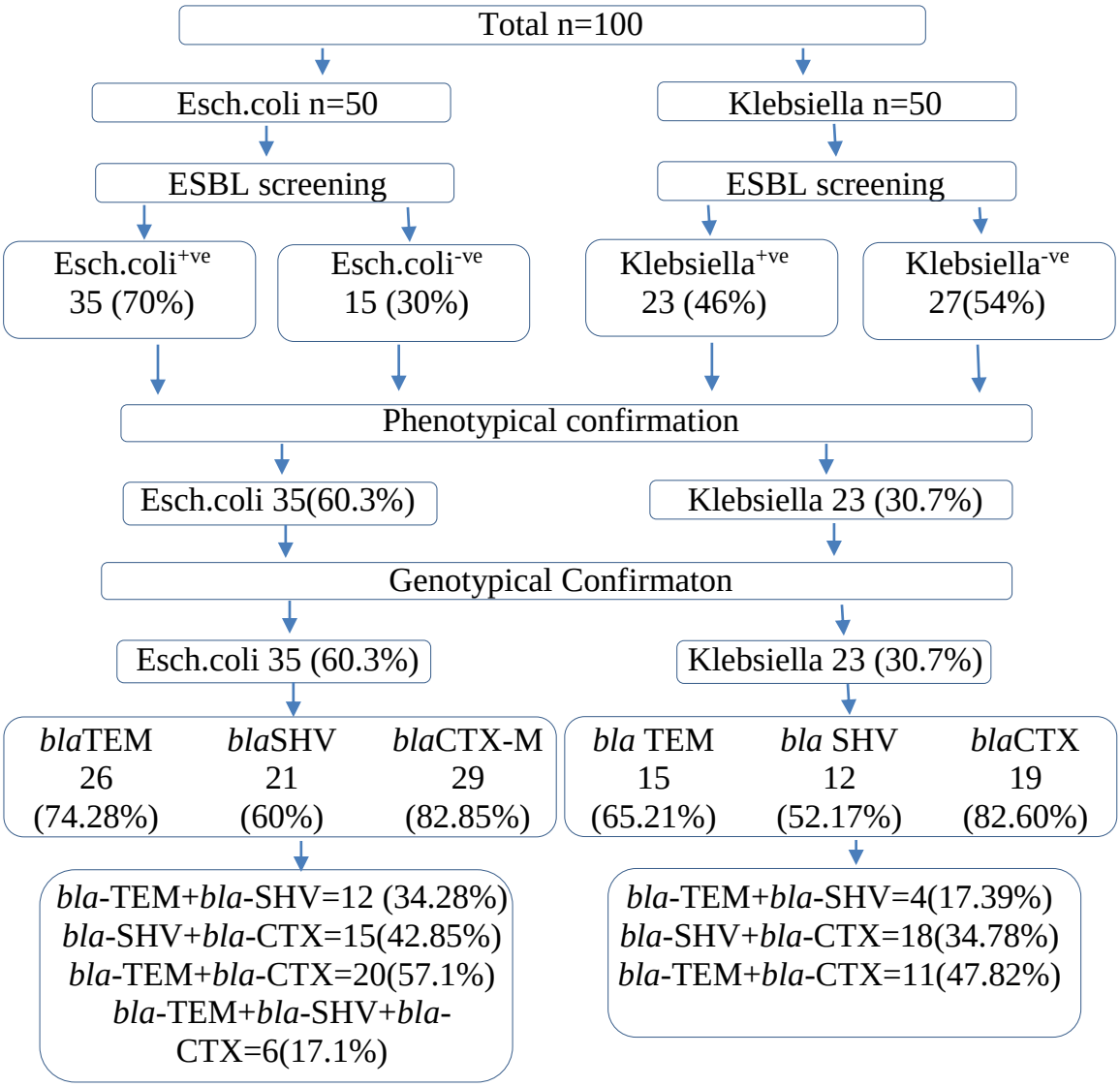
- *bla*-CTX-M is the predominant gene found in the present study which is show a relationship with the studies done previously by Ensor et al 2006, and sirdar PN Rao 20014.[106,110]
- In this study among the 58 ESBL producers Esch.coli 35 60.3% is the predominant ESBL producer and in Klebsiella 23 (30.7%) were ESL producers which correlates with most of the previous studies.[30,81,106,107,108,116.]
- This study has some limitations. ESBLs are reported worldwide among different bacterial species; including Enterobacteriaceae and non-fermentative Gram-negative bacteria such as *Pseudomonas* and *Acinetobacter* species. This study assessed ESBL only with 50 isolates of Esch.coli and 50 isolates of Klebsiella which are the common isolates in our Institute.
- The small sample size may limit also the power of the study to recognize other resistant genes such as AmpC, Carbapenemase and other ESBL-producers.
- Needs further study with more number of samples and detection of other genes will ensure better understanding of ESL producers in the clinical samples in our Institution.

Summary

SUMMARY

- A total of 100 isolates (50 Esch.coli and 50 Klebsiella) were isolated from various clinical samples were studied for ESBL production. In these isolates ESBL was detected by two steps as per CLSI 2010 guideline: Screening by resistant to 3rd Gen. Cephalosporins and Combination disk diffusion using ceftazidim and cefotaxime alone and with clavulanic acid. By phenotypic method, 35/50 (70%) of Esch.coli and 23/50(46%) were ESBL producers. It shows that Esch.coli has high prevalence than Klebsiella.
- When the ESBL producers subjected to antibiotic sensitivity testing they were observed that all the 58 isolates were resistant to 3rd Gen. Cephalosporins such as cefotaxime, ceftriaxone and ceftazidime. At the same time it was found the isolates were resistant to Cefoxitin and Ciprofloxacin.
- More than 70% of ESBL producers were sensitive to Amikacin and Imipenem, it shows that these drugs continue to be effective against ESBL producers.
- With PCR technique, *bla*-TEM, *bla*-SHV, *bla*-CTX genes were detected among 58 isolates 35 Esch.coli and 23 Klebsiella which was confirmed as ESBL producers phenotypicaly.
- *Bla*-CTX-M was the most common gene among Esch.coli and Klebsiella.
- Other two genes *bla*-TEM and *bla*-SHV was detected in more number of Esch.coli than Klebsiella.

Flow chart 3: Summary



Conclusion

CONCLUSION

- The prevalence rate of ESBL producing Esch.coli was significantly higher than Klebsiella isolates in our Institution.
- 2nd and 3rd Generation cephalosporins and ciprofloxacin shows high resistance.
- More than 70% of isolates shows high sensitivity to Amikacin and Imipenem.
- CTX-M gene was detected in more number of Esch.coli and Klebsiella.
- Antimicrobial policy making and strict adherence in the need of the day can prevent drug resistance.
- Epidemiological studies of β-lactamases in each Institute and genetic environment of the clinical isolates would be useful to prevent bacteriological drug resistance.

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PROFORMA

Sree Mookambika Institute of Medical Science, Kulasekharam

DEPARTMENT OF MICROILOGY

Study title:

”Extented spectrum beta lactamases screening in Escherichia coli and Klebsiella isolates and confirmation by molecular method”

Serial Number :

Name :

Age/Sex :

Hospital Number :

Ward No :

Sample type :

Date of collection :

Time of collection :

Sample receiving time :

Anti-biotic usage : Yes/No

If yes details :

Serial Number	Name of the isolate	Frequency	Percentage	
			ESBL +ve	ESBL -ve
1	E.coli			
2	Klebsiella			

Serial Number	Antibiotics	Resistance (%)	Name of the organism	
			E.coli	Klebsiella

CONSENT FORM

PART 1 OF 2

INFORMATION FOR PATICIPANTS OF THE STUDY

Dear Participants,

We welcome you and thank you for your keen interest in participating in this research is being carried out. This form will provide you all the relevant details of this research. It will explain the nature, the purpose, the benefits, the risk, the discomforts, the precautions and the information about how this project will be carried out. It is important that you read and understand the contents of the form carefully. This form may contain certain scientific terms and hence, if you have any doubts or if you want more information, you are free to ask the study personnel or the contact person mentioned below before you give your consent and also at any time during the entire course of the project.

Study title:

"Extented spectrum beta lactamases screening in Escherichia coli and Klebsiella isolates and confirmation by molecular method"

Name of the Principal Investigator:	**Dr.J.Jasmine Gnana SuthA** Post Graduate Student Deportment of Microbiology SMIMS, Kulasekharam
Name of the Guide	**Dr.N.Palaniappan , M.D** Professor Deportment of Microbiology SMIMS, Kulasekharam
Name of the Co-Guide	**Dr.P.Indu, M.D** Professor Deportment of Microbiology SMIMS, Kulasekharam
Institute: Sree Mookambika Institute of Medical Science (SMIMS), Kulasekharam, Kanyakumari District, Tamilnadu	

Background information:

The phenotypical test for identification of ESBL based on disk-diffusion test is the most common strategy used in microbiology laboratories in India. However, this method has serious limitations, as additional resistance mechanisms can cause divergence in the results. Only few studies in India investigated the genotypic origin of ESBL, so there is little knowledge on the epidemiological aspects of the prevalence of these enzymes employing a phenotypic detection procedure based on the combined disk method and a genotypic method based on the detection of blaSHV gene using the polymerase chain reaction (PCR)[3,5,6].

Aims and Objectives:

- To evaluate the prevalence of ESBL producing E.coli and Klebsiella in the clinical isolates at Sree Mookambika Institute of Medical Sciences, Kulasekaram.
- To study the present status of antibiotic resistance in E.coli and Klebsiella.
- To conform the phenotypically resistant E.coli and Klebsiella to genotypes.

Scientific justification of the study:

The incidence of Extended Spectrum Beta Lactamase (ESBL) producing strains among clinical isolates (Klebsiella species and Escherichia coli) has been steadily increasing over the past years. ESBL producing organisms pose a major problem for clinical therapeutics. Identifying organisms that are ESBL producers

are a major challenge for the clinical microbiology laboratory [7]. An attempt to study ESBL production and multidrug resistance in clinical isolates of Klebsiella and E.coli in Sree Mookambika Institute of Medical Sciences, Kulasekaram, Kanykumari district.

Procedure for the study:

The samples will process and isolates will be identify by standard laboratory methods. Antibiotic susceptibility testing will done on Muller Hinton agar by Kirby Bauer's disk diffusion method as per CLSI guidelines. A genotypic method based on the detection of blaSHV gene using the polymerase chain reaction (PCR) [9, 10].

Expected risks for the study:

While collecting blood sample for testing there can be mild pain and bleeding in the site and during pus collection mild tissue injury and bleeding occurs very rarely, and its will be managed easily.

Expected benefits of research for the participants:

This study can help the institution to make a new antibiotic regime which can be ofhelp for better cure.

Maintenance of Confidentiality:

All information for study purpose only. We will not disclosed with others.

Why have I been chosen to be this study?

The incidence of Extended Spectrum Beta Lactamase (ESBL) producing strains has been steadily increasing over the past years. ESBL producing organisms

pose a major problem for clinical therapeutics. To study the present status of antibiotic resistance and to prevent ESBL producing E.coli and Klebsiella at Sree Mookambika Institute of Medical Sciences, Kulasekaram.

1. How many people will be in the study? NA
2. Agreement of Compensation to the participant (In case of a study related injury):NA
3. Anticipated prorated payment, if any, to the Participant(s), of the study: NA
4. Can I withdraw from the study at any time during the study period? NA
5. If there is any new findings/information, would I be informed? NA
6. Expected duration of the Participant's participation in the study: NA
7. Any other pertinent information: NA
8. Whom do I Contact for further information?

For any study related queries, you are free to contact:

Dr. J.Jasmine Gnana Sutha
Post graduate, Department of Microbiology
Sree Mookambika Institute of Medical Sciences,
Kulasekharam, Kanyakumari-629161.
Mobile number: 9487662907,
E-mail: sudhajas@yahoo.com

Place: Kulasekaram

Date: signature of the Principal Investigator

CONSENT FORM

PART 2 OF 2

PARTICIPANTS CONSENT FORM

The details of the study have been explained to me in writing and the details have been fully explained to me. I am aware that the results of the study may not be directly beneficial to me but will help in the advancement of medical sciences. I confirm that I have understood the study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided such a use only for scientific purpose(s). I have been give an information sheet giving details of the study. I fully consent to participate in the study titled **"Extended Spectrum Beta lactamases screening in Escherichia coli and Klebsiella isolates and confirmation by molecular method".**

Name of the participant: Address of the participant:

Contact of the Participant:

Serial no/Reference no:

Signature/Thumb impression of the participant

Witnesses:

Date: 1.

Place: 2.

ஒப்புதல் படிவம்

பாகம் 1 ல் 2

ஆய்வுக்குஉட்படுத்தப்படுபவர்களுக்கானஅறிவுரைகள்

அன்பானநண்பர்களே,

உங்களுடன் ஆய்வுகளை மேற்கொள்வதற்கு நீங்கள் கொடுத்த ஆர்வத்திற்கும் ஒத்துழைப்புக்கும் தங்களை வரவேற்று நன்றியினை தெரிவித்து கொள்கிறேன். இந்த படிப்பின் முக்கியமான சாராம்சங்கள் கீழே கொடுக்கப்பட்டுள்ளது. இதில் கொடுக்கப்பட்டுள்ள ஒவ்வொரு வாக்கியங்களும் மிகவும் முக்கியமான ஒன்றாகும். இதில் ஏதேனும் சந்தேகங்கள் இருப்பின் நீங்கள் கீழ்கண்ட நபர்களை தொடர்பு கொள்ளலாம்.

1. வழிநடத்துபவர் : மரு.ஜாஸ்மின் ஞானசுதா
   முதல் ஆண்டுமுதுநிலைநுண்ணுயிரியல் பிரிவு.
   ஸ்ரீ மூகாம்பிகாமருத்துவகல்லூரி மருத்துவமனை. குலசேகரம்.

2. வழிகாட்டி : மரு.N.பழனியப்பன்

3. இணைவழிகாட்டி : மரு.P.இந்துமதி.

4. கல்விநிறுவனம் : ஸ்ரீ மூகாம்பிகாமருத்துவகல்லூரி மருத்துவமனைகுலசேகரம்.

5. தலைப்பு : Extended Spectrum Beta Lactamases screening in Esherichia coli and Klebsiella isolates and confirmation by molecular method.

6. அடிப்படைவிவரங்கள் :

இந்தியாவில் பொதுவாக நுண்ணுயிரியல் ஆய்வு கூடத்தில் ESBL யின் முக்கியமான பரிசோதனை Disk-Diffusion test. ஆனால் இந்த ஆய்வுமேற்கொள்வதற்கு பிரத்யேகமான வரைமுறைகள் மற்றும் விதிமுறைகள்

உள்ளது. மேலும் இந்த சோதனைகள் மேற்கொள்ளும் போது அதன் முடிவுகள் எப்போதும் ஒரேமாதிரியாக கிடைப்பதில்லை. ESBL யின் ஆய்வினைகுறித்து இந்தியாவில் போதுமான விழிப்புணர்வு இல்லை. அதனால் இந்தியாவில் இந்த ஆய்வு இன்னும் வளர்ச்சி பெறவில்லை. Poly merase chain reaction (PCR) முறையை பயன்படுத்தி blaSHV யினை கண்டுபிடிக்க Phenotypic detection procedure உதவுகிறது.

7. பயன்பாடுகள் :

ய) ஸ்ரீ மூகாம்பிகா மருத்துவ கல்லூரி மருத்துவமனையில் E.coli Klebsilla ன் ESBL உற்பத்தி செய்யும் தன்மையினை ஆராய்தல்.

டி) E.coli Klebsillaவில் நோய் எதிர்ப்புதன்மை குறைவதற்கான காரணம் கண்டறிதல்.

உ) Phenotypically resistanceE.coli Klebsilla-வின் Genotype யினை கண்டறிதல்

8. அறிவியல் விதிமுறைகள்

ESBL உற்பத்தி செய்யும் அணுக்கள் கடந்த வருடத்தை விடவும் அதிகரித்து இந்த நுண்ணுயிர்களை கண்டறிந்து ஆய்வு மேற்கொள்வது மருத்துவதுறைக்கு சவாலான விஷயமாகும். ஸ்ரீ மூகாம்பிகா மருத்துவ கல்லூரி மருத்துவமனையில் E.coli Klebsillaன் ESBL உற்பத்தி செய்யும் தன்மையினை பற்றிய ஆய்வுகள் மேற்கொள்வது நமக்கு சாதகமாகும். இது Clinical Isolate ன் E.coli Klebsillaயில் ESBL உதவியுடன் அதற்குரிய சரியான antibiotics பயன்படுத்துவதற்கான ஆய்வு செய்திட வழிவகுக்கும். ஸ்ரீ மூகாம்பிகா மருத்துவ கல்லூரி மருத்துவமனையில் ESBL யை உற்பத்தி செய்யும் அணுக்கள் அதிகரித்துவரும் சூழலில் Antibiotic resistancy மற்றும் அதனை தடுக்கும் முறைகள் பற்றிகண்டறிதல்.

9. படிப்புமுறை:

இதற்குமாதிரிகள் (sample) சேகரிக்கப்பட்டு அதற்கேற்ற ஆய்வின் மூலம் கண்டறிதல்.

10. இந்த ஆய்வுகளினால் ஏற்படும் கடினம் : ஆய்விற்க்காக இரத்தம், சீழ் எடுக்குகையில் சிறுவலி அல்லது இரத்தகசிவு ஏற்ப்பட வாய்ப்பு உள்ளது. ஆனால் இது எளிதில் சரி செய்திடும் ஒன்றே.

11. இந்த ஆய்வுக்கான நோக்கம் : மருத்துவர்களுக்கு புதிய ஆண்டிபயாட்டிக் முறை அமைப்பதற்க்கும் அதன் மூலம் பயனாளிகளுக்கு தரமான மருத்துவம் கிடைத்திட இந்த ஆய்வு உதவிடும்.

12. இரகசியம் காத்தல் : தகவல்கள் ஆய்வுக்காக மட்டுமே பயன்படத்தபடுகின்றது.

13. இப்படிப்பை படிப்பதன் நோக்கம்

ESBL உற்பத்திசெய்யும் அணுக்கள் கடந்த வருடத்தை விடவும் அதிகரித்து இந்த நுண்ணுயிர்களை கணடறிந்து ஆய்வு மேற்கொள்வது மருத்துவதுறைக்கு சவாலானவிஷயமாகும். ஸ்ரீ மூகாம்பிகா மருத்துவகல்லூரி மருத்துவமனையில் E.coli Klebsillaன் ESBL உற்பத்தி செய்யும் தன்மையினை பற்றிய ஆய்வுகள் மேற்கொள்வது நமக்குசாதகமாகும். இது Clinical Isolate ன் E.coli Klebsillaயில் ESBLஉதவியுடன் அதற்குரிய சரியான antibiotics பயன்படுத்துவதற்கான ஆய்வு செய்திட வழிவகுக்கும்

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இடம்:

ஆய்வுக்குஉட்படுத்தப்படுபவரின் கையொப்பம்

ஒப்புதல் படிவம்

பாகம் 2 ல் 2

ஆய்வுக்கு உட்படுத்தப்படுபவர்களுக்கான ஒப்புதல் படிவம்

இந்த ஆய்வுக்கான விளக்கம் நான் புரியும்படி எனக்கு சொல்லப்பட்டுள்ளது. இந்த ஆய்வு நேரடியாக எனக்கு பயனளிக்காது ஆனால் மருத்துவ படிப்புகளுக்கு உதவியாக இருக்கும் என்பதை தெரிந்து கொண்டேன். ஆய்வு சம்மந்தமான கேள்விகளை என்னிடம் கேட்க நான் சம்மதிக்கிறேன். ஆய்வுக்கு நான் உட்படுத்தப்படுவது லாப நோக்கமற்றது என்றும் இவ்வாய்விலிருந்து எந்நேரமும் விடுவிக்கப்படலாம் என்பதையும் அறிந்துள்ளேன். என்னை உட்படுத்தி கிடைக்கும் ஆய்வு முடிவுகளை மருத்துவ படிப்புக்களுக்காக பயன்படுத்திகொள்ள தடை செய்யமாட்டேன் என்று உறுதியளிக்கிறேன். எனக்கு ஆய்வு பற்றிய விளக்கவுரை வழங்கப்பட்டுள்ளது. நான் "Extended Spectrum Beta Lactamases screening in Esherichia coli and Klebsiella isolates and confirmation by molecular method" என்ற மருத்துவ ஆய்வுக்கு உட்படுத்தப்பட மன்ப்பூர்வமாக சம்மதிக்கிறேன்.

Serial no./ Reference No. :

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ஆய்வுக்குஉட்படுத்தப்படுபவரின் பெயர்: ஆய்வுக்குஉட்படுத்தப்படுபவரின் முகவரி:

ஆய்வுக்குஉட்படுத்தப்படுபவரின் தொடர்புஎண்:

ஆய்வுக்குஉட்படுத்தப்படுபவரின் கையொப்பம்

சாட்சிகள்:

1.
2.

நாள்:

இடம்: